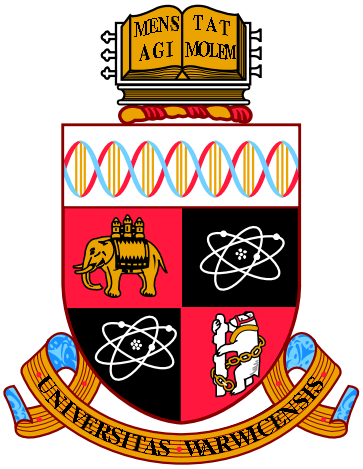

Influence of the circadian clock on Arabidopsis defence against *Botrytis cinerea*

by

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Declarations

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. All of the work in this thesis has been undertaken by myself except where otherwise stated.

Publications resulting from this thesis

Ingle, R. A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carré, I., Roden, L. C. and Denby, K. J. (2015), Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen *Botrytis cinerea*. *Plant J,* **84**, 937–948.

Grundy J., Stoker C. and Carré I. A. (2015). Circadian regulation of abiotic stress tolerance in plants. *Front. Plant Sci.* **6;** 648.

Windram, O., Stoker, C. and Denby, K. (2015) Chapter 17: Overview of Plant Defence Systems: Lessons from Arabidopsis-*Botrytis cinerea* Systems Biology**. In:** S. Fillinger, Y. Elad (eds.), *Botrytis – the Fungus, the Pathogen and its Management in Agricultural Systems*, Springer International Publishing Switzerland.

Abstract

The circadian clock is an endogenous mechanism that provides a wide variety of organisms with the ability to anticipate daily environmental changes. It was shown that under cyclic and constant light growth conditions Arabidopsis exhibits rhythmicity in *Botrytis cinerea* resistance, with maximal resistance observed when leaves were inoculated at dawn. Crucially, this mechanism was confirmed to be under circadian clock regulation. To understand how the circadian clock was driving an effective defence response, genes that were more rapidly induced or repressed after inoculation at dawn compared to night were identified. This indicated a complex interaction between the circadian clock and the defence regulatory network. Phytohormone defence signaling, in particular jasmonate (JA) and ethylene responses, was shown to contribute to the observed rhythmic variation in resistance. This was further confirmed by the identification of a JA signaling mutant (*jaz6*), which displayed no difference in resistance to *B. cinerea* following inoculation at dawn or night under cyclic or constant light conditions. Given the central role of JAZ6 in the circadian defence response against *B. cinerea,* it was likely transcription factors (TFs) bound by JAZ6 were potential links between the plant circadian clock and the defence response. Elucidating the TFs that interacted with JAZ6 revealed JAZ6 to be able to interact with a TF shown to be crucial to the *B. cinerea* defence response, EIN3. Moreover, JAZ6 was also able to interact with a central regulator of circadian clock, FHY3. Both TF interactors indicate JAZ6 is a linking protein between the circadian clock and the defence response against *B. cinerea*.

To further understand how the circadian clock was mediating the plant defence response against *B. cinerea* genome-wide chromatin accessibility data was generated using ATAC-Seq. This aimed to enable the comparison of chromatin accessibility as well as TF binding in regions surrounding genes related to *B. cinerea* defences between the two time-points. This protocol was not optimized for plant tissue. Steps within in laboratory-based ATAC-Seq protocol were therefore pinpointed for tissue specific optimization. Thus, a protocol specific to ATAC-Seq data analysis was proposed.

Abbreviations

ABA	*Abscisic Acid*
35S	*Cauliflower Mosaic Virus Promoter*
3AT	*3-Amino-1, 2, 4-Triazole*
A	*Adenine*
ACC	*1-Aminocyclopropane-1-Carboxylic Acid*
ACS	*Acc Synthase*
AD	*Activation Domain*
Ade	*Adenine*
ADE2	*Yeast Adenine Biosynthesis Reporter Gene*
AGO	*Argonaute Proteins*
AK	*Adenylate Kinase*
AOS	*Allene Oxide Synthase*
ATAC-Seq	*Assay For Transposase-Accessible Chromatin Coupled With High Throughput Sequencing*
Bc	*Botrytis Cinerea*
BcPG	*Botrytis Endopolygalacturonase*
BcPL	*Pectate Lyase*
BcPM	*Pectinmethylesterase*
BcRG	*Rhamnogalacturonase*
BD	*DNA Binding Domain*
bHLH	*Basic Helix-Loop-Helix*
BIFC	*Bimolecular Fluorescence Complementation*
bp	*Base Pair*
bZIP	*Basic Leucine-Zipper Protein*
C	*Cytosine*
CaMV	*Cauliflower Mosaic Virus*
CARM	*CRISPR/Cas9 Assisted Removal Of Mitochondrial DNA*
CBF	*C-Repeat Binding Factor*
CCA1	*Circadian Clock Associated 1*
cDNA	*Complementary DNA*
ChIP	*Chromatin Immunoprecipitation*
ChIP-Seq	*Chromatin Imuuno Precipitation-Sequencing*
Cis-BP	*Catalogue Of Inferred Sequence Binding Preferences*
Co-IP	*Co-Immunoprecipitation*
CO^2	*Carbon Dioxide*
COI1	*Coronatine Insensitive 1*
Col-0	*Arabidopsis Thaliana Columbia Ecotype*
COR	*Coronatine*
CRISPR	*Clustered Regularly Interspaced Short Palindromic Repeats*
CSI	*Causal Structure Identification*
CT	*Circadian Time*
CTR1	*Constitutive Triple Response 1*
DAMP	*Danger Associated Molecular Pattern*
DAMPs	*Damage-Associated Molecular Patterns*

DD	*Constant Dark*
DE	*Differentially Expressed*
DEG	*Differentially Expressed Genes*
DETFs	*Differentially Expressed Transcription Factors*
DHS	*DNase-Hypersensitive Site*
DNA	*Deoxyribonucleic Acid*
DNase-seq	*DNase I Hypersensitive Sites Sequencing*
e.g.	*Exempli Gratia*
EAR	*ERF–Associated Amphiphilic Repression*
EBF	*EIN3 Binding F-Box*
EC	*Evening Complex*
EE	*Evening Element*
EIL1	*Ethylene-Insensitive3-Like 1*
EIN	*Ethylene Insensitive*
ER	*Endoplasmic Reticulum*
ERF	*Ethylene Response Factor*
ERS1	*Ethylene Sensor1*
ET	*Ethylene*
ETI	*Effector-Triggered Immunity*
ETR1	*Ethylene Response 1*
FAIRE-seq	*Formaldehyde-Assisted Isolation Of Regulatory Elements Sequencing*
FAO	*Food And Agricultural Organization*
FHY3	*Far-Red Elongated Hypocotyls 3*
FIMO	*Find Individual Motif Occurrences*
FLD	*Fragment Length Distribution*
flg22	Sequence On Flagellin
FRQ	*Frequency*
G	*Guanine*
GA	*Gibberellic Acid*
GAL4	*Yeast Transcriptional Activator*
GE	*Genome Editing*
GFP	*Green Fluorescent Protein*
GM	*Genetic Modification*
GO	*Gene Ontology*
h	*Hours*
H_2O_2	*Hydrogen Peroxide*
HC	*Hot:Cold*
HDAC	*Histone Deacetylase*
His	*Histidine*
HIS3	*Yeast Histidine Biosynthesis Reporter Gene*
Hpa	*Hyaloperonospora Arabidopsidis*
HPI	*Hours Post-Inoculation*
HR	*Hypersensitive Response*
Hrp	*Hypersensitive Response And Pathogenicity*
ICS1	*Isochorismate Synthase 1*
IGV	*Integrative Genomics Viewer*

INTACT	*Isolation Of Nuclei Tagged In Specific Cell Types*
IS	*Internal Standard*
JA	*Jasmonic Acid*
JA-Ile	*Jasmonate-Isoleucine*
JAR1	*Jasmonate Resistant 1*
JAZ	*Jasmonate ZIM Domain*
KO	*Knock-Out*
LD	*Light Dark*
Leu	*Leucine*
LHY	*Late Elongated Hypocotyl*
LL	*Constant Light*
lncRNA	*Long Non-Coding RNA*
LOX	*Lipoxygenase*
LSD1	*Lesion Simulating Disease Resistance 1*
MAPK	*MAPK Kinase*
MAPQ	*Mapping Quality*
MeJA	*Methyl Jasmonate*
MEME	*Multiple EM For Motif Elicitation*
Mg^{2+}	*Free Magnesium*
miRNA	*MicroRNA*
MKK	*Mitogen-Activated Protein Kinase Kinase*
MKS	*Mitogen-Activated Protein Kinase Substrate*
MPK	*Mitogen-Activated Protein Kinase*
mRNA	*Messenger RNA*
MS	*Mass Spectrometry*
mt	*Mitochondrial*
MTC	*Mutiple Testing Correction*
MYC2	*Myelocytomatosis Related Protein 2*
NADPH	*Nicotinamide Adenine Dinucleotide Phosphate-Oxidase*
NASC	*National Arabidopsis Stock Centre*
NPR	*Non-Expresser Of PR Genes*
nts	*Nucleotides*
O_2^-	*Superoxide*
OGs	*Oligogalaturonides*
OPDA	*12-Oxo-Phytodienoic Acid*
ORF	*Open Reading Frame*
OX	*Overexpressor*
PA-MS	*Pull-Down-Assays Coupled With Mass Spectrometry*
PAD	*Phytoalexin Deficient*
PAMP	*Pathogen-Associated Molecular Patterns*
PCR	*Polymerase Chain Reaction*
PDF	*Plant Defensin*
PG	*Endopolygalacturonase*
PGIP	*BcPG-Inhibiting Proteins*
PHT	*Phosphate Transporter*
PIF	*Phytochrome Interacting Factor*

pmRNA	*Pre-mRNA*
pmRNA	*Premature mRNA*
pt	*Plastid*
PTI	*Pamp Triggered Immunity*
PWM	*Position Weight Matrices*
qPCR	*Quantitative PCR*
R-gene	*Resistance-Gene*
R: FR	*Red: Far-Red*
RGA	*Repressor of ga1-3*
RISC	*RNA-Induced Silencing Complex*
RLKs	*Receptor-Like Kinases*
RNA	*Ribonucleic Acid*
RNA Pol	*RNA Polymerase II*
RNA-seq	*RNA Sequencing*
RNAi	*Ribonucleic Acid Interference*
ROS	*Reactive Oxygen Species*
RT	*Room Temperature*
SA	*Salicylic Acid*
SAR	*Systemic Acquired Resistance*
SCF	*Skp1/Cullin/F-Box*
SD	*Synthetic Dextrose*
SEM	*Standard Error Of The Mean*
sgRNA	*Single Guide RNA*
SNP	*Single Nucleotide Polymorphism*
sRNA	*Small RNA*
SYD	*Splayed*
T	*Thymine*
T-DNA	*Transfer-Deoxyribonucleic Acid*
TCP	*Teosinte Branched Cycloidea And PCF*
TF	*Transcription Factor*
TIC	*Time For Coffee*
Tn5	*Tn5 Transposase*
TPL	*Topless*
TPL-R	*TPL-Related*
tRNA	*Transfer RNA*
Trp	*Tryptophan*
TSS	*Transcription Start Site*
TTFL	*Transcription And Translation Feedback Loops*
UN	*United Nations*
UPLC	*Ultra Performance Liquid Chromatography*
WAK	*Cell Wall-Associated Kinase*
WT	*Wild-Type*
Y1H	*Yeast-1-Hybrid*
Y2H	*Yeast-2-Hybrid*
YPDA	*Yeast Peptone Dextrose Adenine*
ZT	*Zeitgeber Time*

Chapter 1: Introduction

1.1 *Botrytis cinerea*

1.1.1 *B. cinerea* is a threat to the future of food security

United Nation (UN) experts have estimated that food production will need to double by 2050 in order to feed the rising population, which is predicted to reach over nine billion within 35 years (United Nations, 2013). This task will be difficult as the amount of global arable land is decreasing and so higher yield will need to be grown on less land. Furthermore, climate change is increasing temperatures and the frequency of erratic weather patterns, both of which are predicted to make crop production even more challenging (Lobell *et al.,* 2011). Tackling the world's food security crisis will require a multifaceted, collaborative effort. However, there are several ways in which plant pathology may be able to assist.

Worldwide it is estimated that over 25% of crop losses are due to pests or disease (Global Food Security, 2015). Moreover, the UN Food and Agricultural Organization (FAO) has estimated that one third of food produced for human consumption is lost post-harvest (Gastavsson *et al.,* 2011). *Botrytis cinerea* is considered to be the second most important fungal plant pathogen with the ability to infect over 200 crops both pre- and post-harvest (Dean *et al.* (2012); Jarvis (1977); Williamson *et al.* (2007)). Worldwide losses to *B. cinerea* have been estimated to cost between $10-$100 billion per annum (Weiberg *et al.,* 2013).

Control measures against *B. cinerea* vary depending on the plant host, but generally rely heavily upon the use of fungicides. In grapes, *B. cinerea* is a prevalent and economically damaging pathogen. Current control measures integrate fungicidal applications with canopy management (Genescope, 2002). However, control through fungicide usage is becoming increasingly less

effective as *B. cinerea* is developing resistance faster than new fungicides can be brought to market. For example, vineyards in Chile had great success in the 1990s with the dicarboximide fungicides, however this success decreased by 1994 due to the high prevalence of resistant *B. cinerea* strains (Latorre *et al.,* 1994). Then, in the early 2000s, a new class of fungicides (the anilinopyrimidines) was released and successfully reduced losses to *B. cinerea* in Chilean vineyards (Latorre *et al.,* 2002). Nonetheless, by 2002 the prevalence of resistance against the anilinopyrimidines was increasing in Chilean vineyards, despite the implementation of anti-resistance strategies (Latorre *et al.,* 2002). It appears control measures that rely upon fungicides are unsustainable given how quickly fungicide resistance emerges in *B. cinerea* strains.

Moreover, concerns over the impact of fungicides on the environment and human health have prompted a decrease in pesticide availability and usage; this is especially evident in Europe (Clarke *et al.,* 2011). Alternative control measures to combat *B. cinerea* that do not rely upon fungicide usage are therefore becoming increasingly valuable. Hence, a fuller understanding of how a plant naturally defends itself against *B. cinerea* would allow for the genes involved in host defence to be introduced into crops, using methods such as selective breeding, genetic modification (GM) and/or genome editing (GE).

1.1.2 *B. cinerea* as a model necrotroph

B. cinerea is considered an excellent model for studying necrotrophic plant-pathogen interactions. Not only is it a scientifically and economically important pathogen (Dean *et al.,* 2012), it is also easy to propagate in a laboratory environment. In recent years the genetic sequences of several strains have been published, as well as the means to genetically alter specific strains (Amselem *et al.* (2011); Atwell *et al.* (2015); Blanco-Ulate *et al*. (2013); Schumacher *et al.,* (2012); Staats and van Kan (2012)).

B. cinerea was traditionally considered one of the few 'true' necrotrophs as it kills a broad range of host plants in a typically necrotrophic manner and appears to have a relatively simple life cycle compared to other pathogens (Schumacher and Tudzynski, 2012). Fungal enzymes degrade host tissue and the nutrients released from the dead cells are absorbed. Despite its classification as an archetypical necrotrophic pathogen, more recently it has been shown that *B. cinerea* can also present as a biotroph (*B. cinerea* lifestyle is reviewed in van Kan *et al.* 2014).

Depending upon the inoculation conditions *B. cinerea* can present as either a necrotroph or biotroph. *B. cinerea* mycelium have been recovered from inside *Primula* × *polyantha* leaves which displayed no signs of necrosis (Barnes and Shaw, 2003). A similar pattern was seen in cyclamen and lettuce (Sowley *et al.* 2010). In all cases the young plants remained asymptomatic throughout several developmental stages up until the plants reached flowering age. Once plants reached flowering age *B. cinerea* is thought to have switched from a biotrophic to a necrotrophic lifestyle, as this is when necrosis was evident.

Thus, it was hypothesized by van Kan *et al.,* (2014) that *B. cinerea* can switch between the two lifestyles. This switch is thought to be dependent upon an environmental trigger, which maybe linked to plant age.

1.1.3 Life cycle of *B. cinerea*

The overall *B. cinerea* life cycle is broadly similar irrespective of its host (Fig.1.1).

The infection cycle of *B. cinerea* begins with mobile conidiospores attaching to the plant surfaces and germinating to form appressorium-like structures within 10-12 hours of inoculation (Choquer *et al.* (2007); Gwynne-Vaughan (1922); van Kan (2006)). This is followed by the formation of fungal hyphal. Once formed, hyphae secrete cell-wall degrading enzymes such as endopolygalacturonases (BcPGs), and hydrogen peroxide (H_2O_2) prior to host

penetration (van Kan, 2006). Subsequent necrotrophic colonization inside the host tissue is achieved through the secretion of non-specific phytotoxins, disruption of the host redox state by the release of H_2O_2, inhibition of plant antimicrobial products production and release of elicitors to induce host cell death (Govrin *et al.* (2006); van Kan (2006); van Baarlen *et al.* (2007)).

Once *B. cinerea* has colonized plant tissue, it then forms thick 'claw' like structures (Windram *et al.,* 2012). The function of these structures is unknown however they formed between 18-20 hours post inoculation (hpi) in inoculations that occurred at Zeitgeber time (ZT) 6 (Windram *et al.,* 2012). ZT refers to hours after sunrise, or in this context, then lights in the incubation cabinets come on. Previous research has speculated 'claw' structure formation plays a role in penetration of host tissue (Kunz *et al.,* 2006).

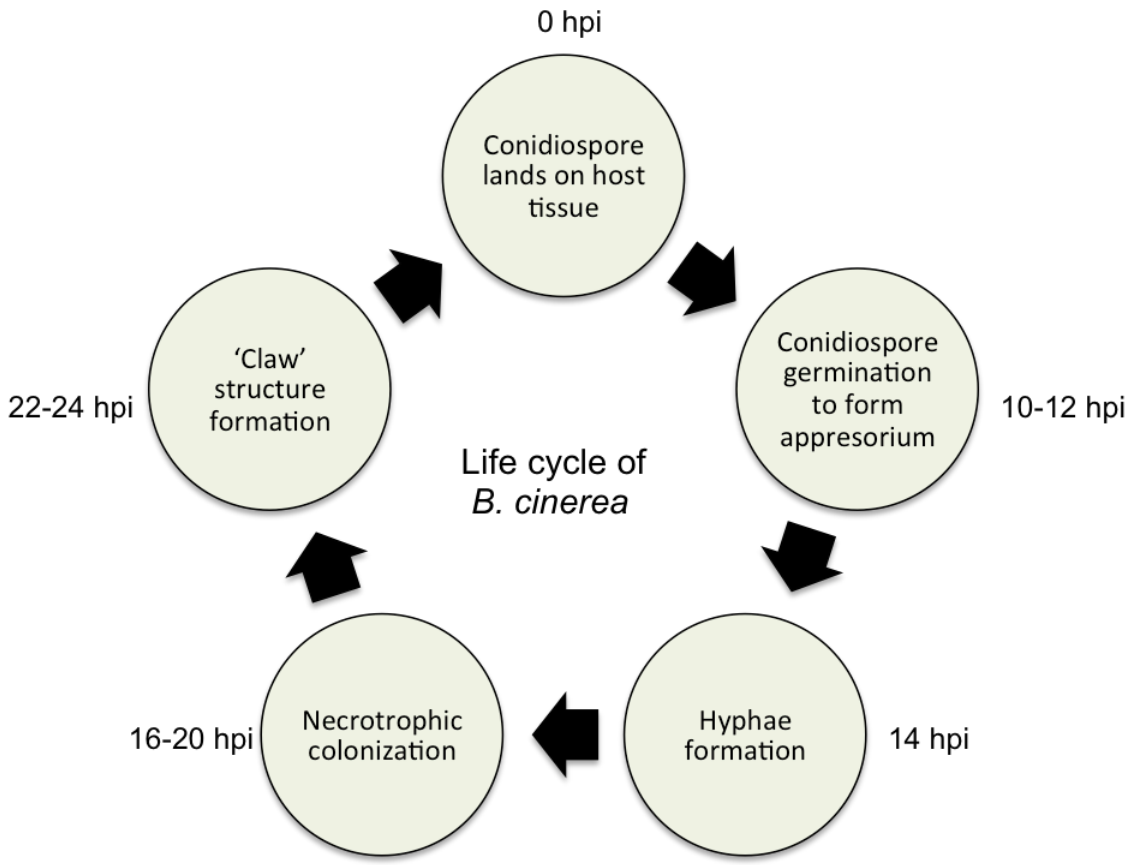


Figure 1.1 - *Botrytis cinerea* infection cycle on Arabidopsis leaves - *B. cinerea* infection stages on 4-week-old *A. thaliana* leaves under the conditions outlined in methods section. Trypan blue staining and microscopy determined infection stages. hpi -hours post inoculation.

1.1.4 Virulence factors

As a primarily necrotrophic pathogen, *B. cinerea* feeds on dying plant cells to gain nutrients. Hence, it is in the fungus' best interest to quickly enter and kill the plant tissue. To this end, *B. cinerea* uses several different infection mechanisms, which include the production of reactive oxygen species (ROS) and a vast array of proteins and toxins to break down the plant cell wall and induce cell death.

1.1.4.1 *B. cinerea* decomposes the host cell wall

The first challenge *B. cinerea* faces when presented with its plant host is penetration of the cell wall. To enter cells *B. cinerea* secretes a myriad of cell wall disrupting enzymes and proteins, which allows the pathogen to release and consume the nutrients trapped in the cell walls as well as penetrate the rest of the cell.

Plant cell walls are made up of a complex network of celluloses, pectins and structural proteins. *B. cinerea* targets each of these components, with pectins being the primary target (Blanco-Ulate *et al.,* 2014). Pectins are degraded by *B. cinerea*-produced BcPGs, rhamnogalacturonases (BcRGs), pectinmethylesterases (BcPMs) and pectate lyases (BcPLs) (Chen *et al.* (1997); Kapat *et al.* (1998); Schols *et al*. (1990); Wubben *et al.* (2000)). Several of these enzymes have shown vital roles in virulence; two (BcPG1 and BcPG2) of the six studied BcPGs are vital for successful *B. cinerea* colonization of host tissue (Kars *et al*. (2005); Have *et al.* (1998); Williamson *et al.* (2007)). Moreover, two BcPMs (*Bcpme1* and *Bcpme2*) have also been mutated and their role in *B. cinerea* virulence determined, (Kars *et al.* (2005); Valette-Collet *et al*. (2003)). The *Bcpme1* knockout was investigated in two *B. cinerea* strains (B05.10 and Bd90); in the B05.10 strain virulence was not influenced (Kars *et al.*, 2005), however a knockout in the Bd90 strain significantly reduced virulence (Valette-Collet *et al.,* 2003). BcPGs and fragments of the degraded cell wall are then

sensed by plant receptors, which then trigger further immune responses.

1.1.4.2 *B. cinerea* produces ROS to trigger host cell death

ROS release is not only a plant defence mechanism but is also a pathogenicity mechanism for *B. cinerea*. ROS such as superoxide (O_2^-) and H_2O_2 generation occurs at the point when the host is penetrated by the fungus with both fungal (Schouten *et al.,* 2002) and plant enzymes contributing to this ROS release*. B. cinerea* exploits the host immune response as it induces ROS release to trigger cell death via the plant hypersensitive response (HR) (Govrin *et al.,* 2000). Arabidopsis lines with delayed or reduced cell death responses have been shown to have reduced *B. cinerea* disease progression, and conversely, plants with an increased cell death response were more susceptible to *B. cinerea* (Van Baarlen *et al.,* 2007).

B. cinerea possesses two Nicotinamide Adenine Dinucleotide Phosphate-Oxidase (NADPH) oxidases, BcNoxA and BcNoxB, which are transmembrane proteins capable of ROS production (Bedard and Krause, 2007). BcNoxA and BcNoxB are involved in *B. cinerea* virulence; BcNoxA is crucial to host colonization (Segmüller *et al.,* 2008) and BcNoxB is crucial for host penetration via the appressoria (Siegmund *et al.,* 2013).

1.1.4.3 *B. cinerea* produces various phytotoxic compounds to kill plant cells

B. cinerea produces several known phytotoxic terpenes and polyketides, including the terpenes botrydial and botryane derivatives, and the polyketides botcinic acid, botcinins and botrylactone (Collado *et al.* (2000); Collado *et al.* (2007); Shiina and Fukui (2009)). It is hypothesized that many of these compounds are effective against a broad range of host plant cells.

Isolated botrydial can independently induce chlorosis in tobacco (Rebordinos *et al.,* 1996) and bean leaves (Colmenares *et al.,* 2002), such chlorotic lesions are

characteristic of a *B. cinerea* infection. Although the specific plant target of botrydial remains to be elucidated, it has been shown that this compound can induce HR by manipulating hormonal pathways in Arabidopsis (Rossi *et al.,* 2011). When genes known to play crucial roles in botrydial biosynthesis (*BcBot1* and *BcBot2*) were inactivated in a *B. cinerea* T4 strain, fungal virulence was severely reduced (Siewers *et al.,* 2005). However, the same mutations in a B05.10 or a SAS56 strain did not influence virulence (Pinedo *et al.,* 2008). This was attributed to the B05.10 and SAS56 strains redirecting resources from botrydial synthesis to botcinic acid synthesis when genes for botrydial synthesis were mutated (Pinedo *et al.,* 2008).

Botcinic acid can also induce cellular chlorosis in a wide range of plant species (Cutler e*t al*. (1993); Cutler *et al.* (1996)). Deletion of both genes involved in botcinic acid biosynthesis (*Bcboa6* or *Bcboa9*) resulted in greatly reduced virulence when compared with wild-type (WT) *B. cinerea* strains (Dalmais *et al.,* 2011).

1.1.4.4 *B. cinerea* manipulates the host defence response

Pathogens, have evolved secretory effector molecules that act to suppress a branch of plant immunity, PAMP-triggered immunity (PTI), PTI is further elaborated on in Section 1.2. Following suppression of PTI, effectors are detected by host intracellular resistance (R) proteins and initiate effector-triggered immunity (ETI) (reviewed extensively in Hogenhout *et al.,* 2009). Pathogens such as the hemi-biotrophic pathogen, *Pseudomonas syringae*, the obligate biotrophic pathogen, *Hyaloperonospora arabidopsidis* (*Hpa*) and the oomycete pathogen, *Phytophthora infestans* have advanced secretion systems which deliver effector proteins into the host cells to suppress defences and increase disease symptoms (Alfano (2009); Bardoel *et al.,* (2011); Cunnac *et al.,* (2009); Pel *et al.,* (2014); Pieterse *et al.,* (2011)). However, effector proteins have yet to be uncovered from *B. cinerea* although this necrotroph does secrete small RNAs (sRNAs) to silence specific messenger RNAs (mRNAs) involved in the host defence response (Weiberg *et al.,* 2013). *B. cinerea* also secretes

abscisic acid (ABA) to manipulate the plant defence pathway and suppress phytohormone defences against necrotrophic pathogens (El Oirdi *et al.,* 2011).

sRNAs are generally between 21-24 nucleotides (nts) long and induce the silencing of target complementary DNA sequences. sRNAs bind to Argonaute (AGO) proteins and guide the RNA-induced silencing complex (RISC) to the target sequences for subsequent degradation. Micro RNAs (miRNAs) are a class of sRNAs that have been shown to function as regulatory mechanisms within plants to defend against pathogen attack.

This rapid gene regulatory mechanism has been evolutionarily exploited by plant pathogens. *B. cinerea* secretes sRNAs into plant cells to silence specific mRNAs involved in the host defence response (Weiberg *et al.,* 2013). *B. cinerea* B05.10 has been shown to secrete sRNAs into Arabidopsis and *Solanum lycopersicum* tissue, these sRNAs hijack host RNA silencing machinery (AGO proteins) to suppress genes key to the host *B. cinerea* defence response (genes such as *mitogen activated protein kinase 2 (MPK2)* and *MPK1,* an oxidative stress-related gene, peroxiredoxin (*PRXIIF*) and cell wall-associated kinase (*WAK*)) (Weiberg *et al.,* 2013). Plant mutants lacking in *AGO1* expression showed no reduction in expression levels of the aforementioned defence related genes (Weiberg *et al.,* 2013). In contrast, plants constitutively overexpressing *B. cinerea* sRNA constructs were significantly more susceptible to *B. cinerea* infection compared to WT (Weiberg *et al.,* 2013).

It is generally accepted jasmonic acid (JA) and ethylene (ET) promote plant defences against necrotrophic pathogens and this pathway is suppressed by salicyclic acid (SA), which promotes plant defences against biotrophic pathogens (Glazebrook, 2005). However, this is an over-simplification (see section 1.2.2).

The role of abscisic acid (ABA) in biotic defences is still not fully understood, it appears to act as both a positive and negative regulator of pathogen defence depending upon experimental conditions. Arabidopsis mutants with increased

ABA levels have enhanced immunity to the hemi-biotroph, *P. syringae* (Fan *et al.,* 2008). However mutants with reduced ABA levels had increased immunity to the biotrophic pathogens, *H. parasitica* and *Blumeria graminis* (Jensen *et al.* (2008); Mohr and Cahill (2003)), the necrotroph, *B. cinerea* (Audenaert *et al.,* 2002) and reduced immunity to other necrotrophic pathogens such as *A. brassicicola and Pythium irregulare.* (Adie *et al,* (2007); Ton and Mauch-Mani (2004)).

ABA deficient tomato mutants are more susceptible to *B. cinerea* infection (Audenaert *et al.,* 2002). However, Arabidopsis ABA deficient or ABA insensitive mutants showed decreased susceptibility to colonization to *B. cinerea* (Adie *et al.,* 2007). Moreover, pre-treatment of Arabidopsis plants with ABA has been shown to both repress or enhance callose deposition in response to PAMP treatment, this regulation is dependent upon the abiotic growth conditions such as nutrient availability (Luna *et al.,* 2011). Whether ABA is a positive or negative regulator of this defence process is therefore still debated. However, it does appear that ABA is a negative regulator of the defence response to *B. cinerea* in Arabidopsis given that JA and ET responsive genes are upregulated when *ABA DEFICIENT 1 and 2* (*ABA1* and *ABA*2) are mutated (Anderson *et al.,* 2004). Additionally, the exogenous application of ABA suppressed the JA/ET marker gene *PDF1.2* (Adie *et al.* (2007); Anderson *et al*. (2004)). Sivakumaran *et al.,* (2016) referred to ABA as 'a susceptibility factor' in plant pathogen interactions.

Several mechanisms have been proposed to explain the role of ABA in compromising plant immunity, including ABA interacting with the SA pathway (Audenaert et al., 2002), ABA playing a role in H^2O^2 production and cell wall modifications (Asselbergh et al., 2007) and/or ABA suppressing the plant ROS response (L'Haridon et al., 2011).

ABA is synthesized by *B. cinerea* (Marumo *et al.,* 1982; Siewers *et al.,* 2006). This synthesis raises the question as to whether *B. cinerea* uses ABA as an effector molecule to manipulate the host defence response.

Many pathogens deliver effectors into host cells to suppress defences and generally these effectors are proteins (Rafiqi *et al.* (2012); Weiberg *et al.* (2013)). However, *B. cinerea* employs sRNAs and synthesis ABA which likely act as effectors to suppress host immunity in a dynamic way and as yet unknown way.

How the plant hosts defend themselves against such a formidable pathogen as *B. cinerea* will now be discussed.

1.2 Plant defence against pathogens

Plants have evolved highly specialized, multilayered and complex defence systems to defend themselves against frequent pathogen infection. Plant colonization by pathogenic microorganisms is relatively rare with most plant species displaying at least partial resistance to full microbial species (Gurr and Rushton (2005); Heath (1991); Hein *et al.* (2009); Ingle *et al.* (2006)). An entire plant species can be resistant to a complete microbial species; this is termed non-host resistance (NHR) (Heath (1981a); Ingle *et al*. (2006)). Members of a susceptible host plant species can evolve to become resistant to a pathogen, this is termed cultivar resistance (Heath, 1981b). NHR is the most widespread form of plant disease resistance and relies on constitutive preformed barriers such as thick waxy cuticles and plant cell walls, as well as inducible defences (Ingle *et al.,* 2006). These inducible defences depend upon the plant detecting molecules associated with pathogen infection, the so-called pathogen-associated molecular patterns (PAMPs), and host damage-associated molecular patterns (DAMPs).

Initial pathogen detection relies on the recognition of PAMPs, which are conserved molecular sequences within the pathogen, for example, chitin (Boller and Felix, 2009). DAMPs can also be detected by the host, these are generated

by the host in response to harm caused by the pathogen, for example cell wall fragments resulting from cell wall degradation (Hahn *et al.,* 1981). The detection of these PAMPs and/or DAMPs initiates PAMP Triggered Immunity (PTI). PTI is a first line response and quickly implements changes in plant physiology in an attempt by the plant to prevent colonization by the pathogen. More information on both PAMPs and DAMPs can be found in section 1.2.1.1.

1.2.1 Plant defence against *B. cinerea*

There is no evidence to shown that ETI is an important part of the host defence response against *B. cinerea*. No *R*-genes have been uncovered in hosts to indicate that *B. cinerea* secretes effectors. Moreover, a mechanism by the plant to prevent the action of the *B. cinerea* sRNAs has not been uncovered.

Plants have constitutive and inducible responses to protect from *B. cinerea* colonization. Constitutive defences include physical barriers such as waxy cuticles and cell walls, which can protect against hyphal penetration (Goetz *et al.* (1999); Sarig *et al.* (1998)). The cell wall also contains antimicrobial compounds such as polyphenols and phytoalexins (Prusky *et al.,* 2013). However, when these constitutive defences are bypassed by the pathogen, the plant triggers induced responses.

1.2.1.1 *B. cinerea* perception and initial signalling responses

Induced responses are dependent upon the host detecting the pathogen. The host perception and signalling pathways to *B. cinerea* infection are illustrated in Fig.1.2. *B. cinerea* is recognized by the plant cell via the detection of *B. cinerea* PAMPs and host DAMPs generated in response to the fungal attack. PAMPs recognized by the plant include the key component of the fungal cell wall, chitin and cell wall damaging enzymes, BcPGs, which are perceived by the RLK Chitin Elicitor Receptor Kinase 1 (CERK1) (Miya *et al.* (2007); Wan *et al.* (2008)) or the RLK Responsiveness To Botrytis Polygalacturonases 1 (RPBG1) (Zhang *et al.,* 2014), respectively (Fig.1.2). As mentioned, BcPGs break down pectin in plant cell walls; in response plants release BcPG-inhibiting proteins (PGIPs) in

attempt to limit the action of BcPGs (Fig.1.2). The cell wall degrading action of BcPGs releases host oligogalaturonides (OGs) which are recognized as DAMPs by the Wall Associated Kinase 1, WAK1 (Brutus *et al.,* 2010).

Upon perception of *B. cinerea* via PAMP or DAMP recognition, signals are relayed from receptors to downstream components of the signalling pathway by RLKs. Suppressor Of Bir 1 (SOBIR1) is an example of a membrane bound RLK whereas Botrytis Induced Kinase 1 (BIK1) is localized to the cytoplasm. The PAMP receptor, RPBG1, interacts with SOBIR1 (Zhang *et al.,* 2014), which has been shown to be vital for host defence against *B. cinerea* as mutations in SOBIR1 render plants more susceptible to *B. cinerea* (Zhang *et al.,* 2013a). The chitin receptor, CERK1 interacts with BIK1, this interaction causes BIK1 to leave the receptor and move towards cytoplasmic proteins involved in the MAPK signalling cascade (Lu *et al.,* 2010). BIK1 is also essential to *B. cinerea* defences with mutants of *BIK1* displaying enhanced susceptibility to *B. cinerea* (Liu *et al.,* 2013b).

The MAPK signalling cascades are essential to plant defence against a diverse range of pathogens and plays a key role in PTI. RLKs initiate the MAPK signalling cascade. This cascade begins with MAPK kinase kinases (MAPKKK) being activated and phosphorylating a downstream MAPK kinase (MAPKK), MAPKK then activates and phosphorylates final MAPKs. *flg22* is a 22-amino acid conserved sequence on flagellin and is the sequence detected by plant receptors. *flg22* treatment activates MPK3/4/6 (Droillard *et al.,* 2004). The final MAPK then elicits a transcriptional response in the plant cell.

For example, upon activation MAPKs then act upon proteins to elicit PAMP related defences. Mutants reduced in the expression of *MPK3* and *MPK6* show decreased camalexin accumulation in response to *B. cinerea* inoculation (Ferrari *et al.,* 2007). Reduced camalexin levels are associated with decreased resistance to *B. cinerea* as the plant uses camalexin as an antifungal compound (Ferrari *et al.,* 2007). Accordingly, single *mpk3* mutants display enhanced susceptibility to *B. cinerea* (Ren *et al.,* 2008). MPK6 has been shown to be

involved in the OG induced pathway, with *mapk6* mutants showing decreased OG-induced resistance to *B. cinerea* compared to WT (Galletti *et al.,* 2011). MPK4 is also a key player in the defence response (Rasmussen *et al.,* 2012). MPK4 has been implicated in the salicyclic acid (SA) and jasmonic acid (JA) cross talk (further details on the roles of hormones in plant defence in 1.2.2). *mpk4* mutants show enhanced SA responses and reduced JA/ET responses (Brodersen *et al.* (2006); Peterson *et al.* (2000)). As such *mpk4* mutants show decreased resistance to *A. brassicicola*, which depends on both ET/JA pathways (Brodersen *et al.*, 2006). Moreover, resistance to *B. cinerea* is decreased in plants with inactivated MPK4/6 (Schweighofer *et al*., 2007).

How signalling between MAPK pathways and transcriptional regulation is achieved is still largely unknown. It has, however, been established that MPK4 interacts with a MAP Kinase 4 Substrate 1 (MKS1) substrate and that this complex also includes the transcription factor (TF) WRKY DNA-Binding Protein 33 (WRKY33) (Andreasson *et al.,* 2005). Upon infection, MKS1 and WRKY33 are released from the trimer and then positively regulate the defence response by activating camalexin biosynthetic genes such as *PHYTOALEXIN DEFICIENT3 (PAD3)* (Qiu *et al.,* 2011) as well as repressing the transcription of negative regulators of the JA hormonal signalling pathway (JAZ1/5) (Birkenbihl *et al.,* 2012). However, *MKS1-OX* lines increase susceptibility to *B. cinerea*, so *MKS1* may also be able to act as a negative regulator of the defence response against *B. cinerea* (Fiil *et al.,* 2011).

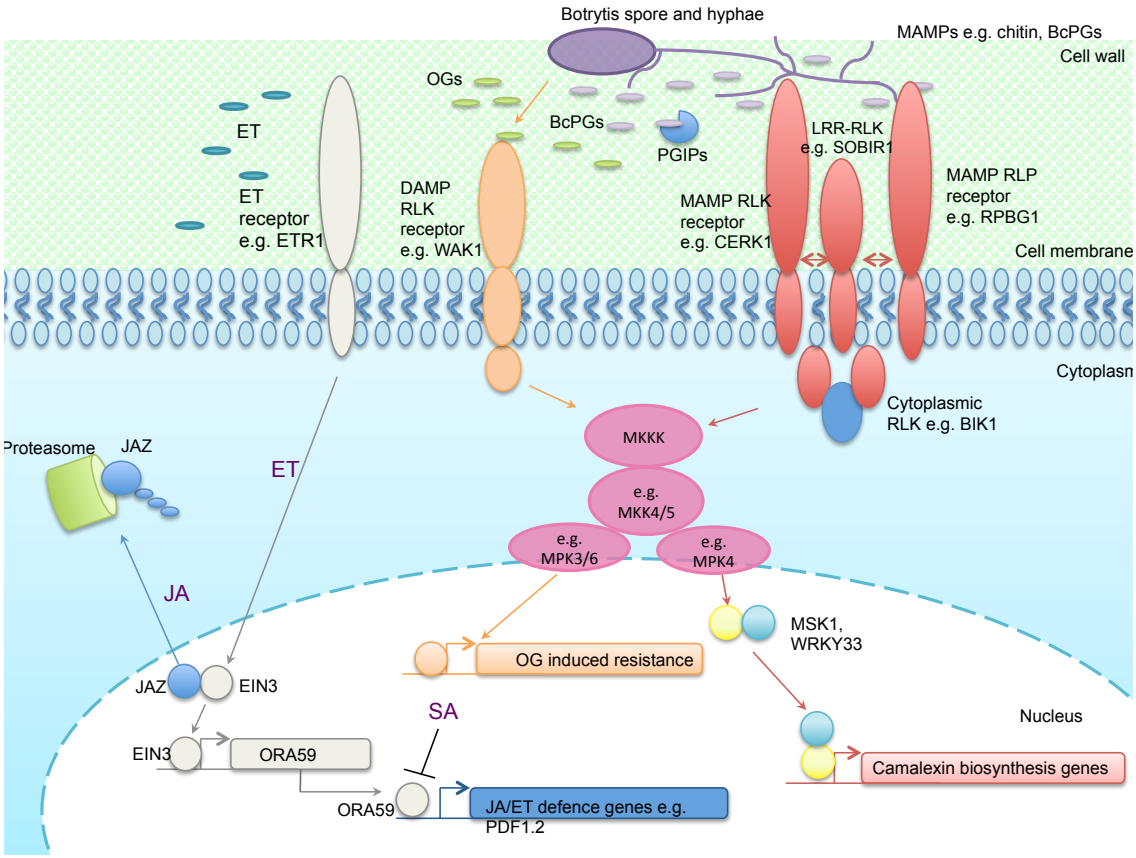


Figure 1.2 - Perception of the pathogen and initial signaling events during *B. cinerea* infection. Examples of microbe-associated molecular patterns (*MAMPs*) and damage-associated molecular patterns (*DAMPs*) present during *B. cinerea* infection are shown. Typical signaling pathways are shown indicating components that are known to play a role in the response to *B. cinerea* infection. MAMPs and DAMPs are perceived by receptor-like kinases (*RLKs*) and receptor-like proteins (*RLPs*) which typically associate with additional leucine-rich repeat (*LRR*) RLKs and/or cytoplasmic RLKs to transduce a signal to kinase cascades. Mitogen activated protein kinase (*MAPK*) kinase kinase (*MAPKKK*) phosphorylate MAPK kinases (*MAPKK*) which in turn phosphorylate MAPKs. MAPKs have been shown to be essential for DAMP-induced resistance and, in the case of MPK4, for activation of the transcription factor WRKY33 by phosphorylation of the MPK4- WRKY33-MPK substrate 1 (*MKS1*) complex. WRKY33 activates expression of camalexin biosynthetic genes, an important resistance determinant against many isolates of *B. cinerea*. Cross talk between the phytohormones ethylene (*ET*), salicylic acid (*SA*) and jasmonic acid (*JA*) is crucial in host defence responses and an example of this is outlined. After infection by *B. cinerea*, production of ET stabilizes the transcription factor (TF) EIN3. Production of JA triggers degradation of the repressive JAZ proteins by the proteasome, allowing the transcriptional cascade of JA and ET related defence genes downstream of EIN3 to proceed. SA represses this transcriptional cascade by inhibiting accumulation of ORA59 protein. In this diagram → indicates positive regulation and ⊣ indicates negative regulation (Reproduced with permission from Windram *et al.,* 2015)

1.2.2 Role of phytohormones in plant defence

Phytohormones such as SA, JA, ethylene (ET) and ABA are known to mediate the plant defence response against abiotic and pathogen induced stress (Bari and Jones, 2009). Generally SA, ET and JA are involved in plant defence against pathogens whereas ABA is more involved in abiotic tolerance. Indeed, it has been shown the concentration of individual hormones can modulate the balance between plant immunity and plant defence (Walters and Heil, 2007). It is however becoming more evident that under pathogen induced stress; hormonal pathways share a high level of cross talk, which is dependent upon many factors including the lifestyle of the pathogen, environmental stresses and the host microbiome. This cross talk involves more than SA, ET, JA and ABA with hormones such as gibberellic acid (GA) and auxin contributing to the defence response (as reviewed in Robert-Seilaniantz *et al.,* 2011).

1.2.2.1 Ethylene

ET is a gaseous hormone that plays a variety of roles throughout the plant, from seed germination to senescence to biotic and abiotic responses (Ruduś *et al.,* 2013).

The Arabidopsis genome encodes five ET receptors; Ethylene Response 1 (ETR1), Ethylene Response Sensor 1 (ERS1), ETR2, ERS2, and Ethylene Insensitive 4 (EIN4) localised to the endoplasmic reticulum (ER) (Lin *et al.* (2009); O'Malley *et al.* (2005); Wang *et al.* (2006)). ET is perceived by ETR1 (or a similar receptor) and this perception inactivates a negative regulator of the ET signalling pathway (Constitutive Triple Response 1 (CTR1)) (Gao *et al.,* 2003). CTR1 inactivation results in ET activation of the MAPK signalling cascades (Gao *et al.,* 2003). The perception of ET also reduces the expression of genes encoding for F-box proteins Ein3-Binding F Box Protein 1 or 2 (EBF1/2) that are hypothesized to suppress *EIN3* activity when ET levels are low, whereas when ET levels rise these proteins are ubiquitinated and targeted for

proteasomal degradation (An *et al.,* 2010). This inactivation relieves key ET signalling genes of their repression. Such genes include the TFs EIN2 and EIN3 which act downstream of CTR1 (Guo and Ecker (2003); Roman *et al.* (1995)). In response to ethylene EIN2, an ER-bound protein, is translocated to the nucleus to active EIN3 expression (Ju *et al.,* 2012; Qiao *et al.,* 2012; Wen *et al.,* 2012). EIN3 can then positively mediate the expression of genes such as *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59* (*ORA59)* and *ETHYLENE RESPONSE FACTOR 1 (ERF1)* (Fig.1.2).

1.2.2.1.1 Role of ethylene in *B. cinerea* defence

The study by Windram *et al.,* (2012) gave great insight into the role of ET in *B. cinerea* infection. Four-week-old Arabidopsis leaf tissue was inoculated with *B. cinerea* strain pepper at Zeitgeber (ZT) 6 (six hours after dawn which in this context is when lights came on in the cabinet) and leaves were then harvested at two hour increments for 48 h. Tissue was then subject to transcriptomic profiling and DE genes (DEGs) between mock and infected tissue were analysed (Windram *et al.,* 2012). Gene ontology (GO) term analysis of groups DEGs can be seen in Fig.1.3.

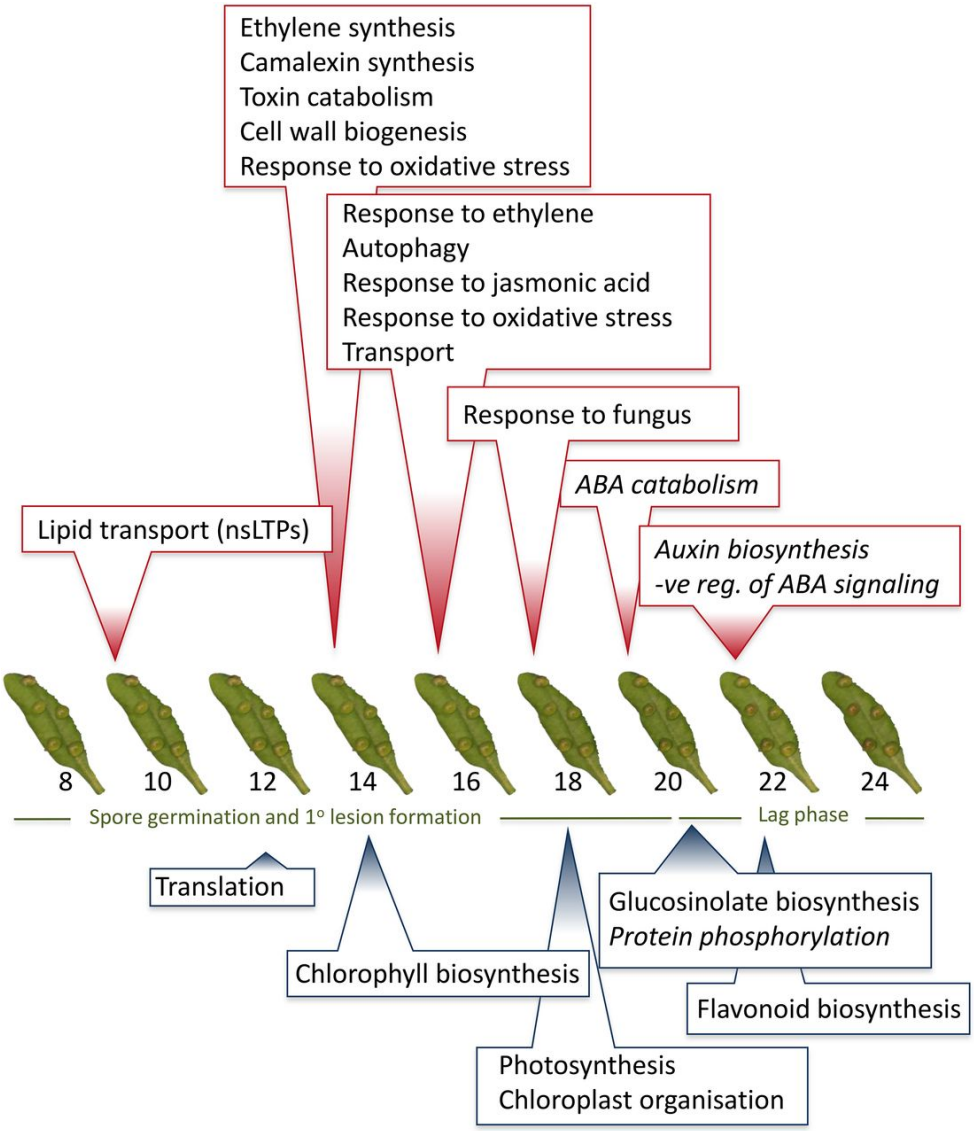


Figure 1.3- Selected Gene Ontology (GO) Terms Overrepresented in Clusters of Genes Differentially Expressed after *B. cinerea* Infection of Arabidopsis Leaves. GO terms are aligned with the time of gradient change and/or time of first differential expression of the cluster (in italics), with red boxes containing GO terms from upregulated genes and blue boxes containing GO terms from downregulated genes. (Reproduced with permission from Windram *et al.,* 2012)

DEGs upregulated in response to infection at 14 hours post inoculation (hpi) were significantly overrepresented for ET biosynthetic processes (Fig.1.3). This cluster also contained genes encoding for enzymes crucial to ET biosynthesis, aminocyclopropane-1-carboxylate synthases (ACS2 and ACS6) (Windram *et al.,* 2012). The cluster of genes upregulated at 16 hpi was subsequently overrepresented for genes associated with ethylene responses. The role of ET in host defence against *B. cinerea* is highlighted in (Fig.1.2). ET is synthesized and

perceived by receptors; these receptors relay the signal to downstream TFs such as EIN3. *ein3-1* mutants have previously been shown to be more susceptible to *B. cinerea* inoculation compared to WT (Zhu *et al.,* 2011). EIN3 then activates the transcription of genes encoding for TFs such as *ORA59* and *ERF1*, which have positive roles in upregulating genes involved in the *B. cinerea* defence response. The role of ERF1 and ORA59 in *B. cinerea* host defence was affirmed when overexpression lines of both *ERF1* and *ORA59* displayed enhanced resistance to *B. cinerea* (Berrocal-Lobo *et al.* (2002); Pré *et al.* (2008)).

1.2.2.2 Jasmonic acid

JA is involved in a wide range of processes in plants from development to fertility and abiotic and biotic responses (Robert-Seilaniantz *et al.,* 2011). It is the most important hormone in defence against necrotrophic pathogens (Penninckx *et al*. (1998); Thomma *et al.* (1998)). JA insensitive mutants (*coi1*) have been shown to be more susceptible to *B. cinerea* and *Alternaria brassicicola* (Thomma *et al.,* 1998).

JA originates from plastid membrane α-linolenic acid and the pathway to synthesise JA was revealed in a study of the *coi1* mutants (Turner *et al.,* 2002), since then the process has been extensively reviewed (Acosta and Farmer (2010); Browse (2009); Kombrink (2012); Schaller and Stintzi (2009); Wasternack (2007); Wasternack and Kombrink (2010)). Once synthesised, JA and the JA derivative, methyl jasmonate (MeJA), can be converted to the active form of JA, by conjugating with isoleucine (Ile) to form JA-Ile. Jasmonoyl Isoleucine Conjugate Synthase 1 (JAR1) facilitates this conjugation (Staswick and Tiryaki, 2004). JA-Ile then serves as the ligand of CORONATINE INSENSITIVE 1 (COI1), which modulates the degradation of jasmonate ZIM-domain (JAZ) repressor complexes (Fonseca *et al.,* 2009) (Fig.1.4). JAZ proteins repress TFs involved in activating JA signalling pathways; once degraded via the COI1 complex, TFs are available to regulate the JA signalling pathway.

1.2.2.2.1 JAZ proteins

There are at least 12 JAZ repressor proteins encoded in the Arabidopsis genome (Chung *et al.,* 2010). Each contains the highly conserved Jas domain, which is essential to the repressive action of JAZ proteins as it mediates binding between JAZ proteins and TFs (Chini *et al.* (2007); Chung *et al.* (2009)). JAZ proteins also contain a conserved ZIM (TIFY) domain, which mediates JAZ to JAZ protein binding interactions as well as interactions between JAZ and Novel Interactor of JAZ (NINJA) (Vanholme *et al.,* 2007) (Fig.1.4).

Under conditions of low JA-Ile, JAZ proteins repress the JA defence pathway by binding to key TFs such as Myelocytomatosis Related Protein 2 (MYC2) (Fernandez-Calvo *et al.,* 2011) (Fig.1.4 and Fig.1.5). JAZ proteins repress TFs by recruiting proteins such as NINJA (Pauwels *et al.,* 2010). NINJA then recruits the repressors TOPLESS (TPL) and TPL-related (TPL-R) proteins; the ERF–associated amphiphilic repression (EAR) domain on NINJA, mediates this recruitment (Pauwels *et al.,* 2010) (Fig.1.4). Repression can also happen via JAZ proteins directly recruiting TPL and TPL-R proteins as four of the 12 JAZ proteins have EAR domains, including JAZ5/6/7/8 (Kagale *et al.* (2010); Shyu *et al.* (2012)). TPL and TPL-R repress gene expression by recruiting histone deacetylases (HDACs), which change the chromatin conformation making that area less accessible to transcriptional activators (Kieffer *et al.,* 2006; Long *et al*., 2006; Zhu *et al*., 2010).

Once JA-Ile levels increase, the repression facilitated by the JAZ proteins is relieved via the ubiquitin-proteasome system (Fig.1.4 and Fig.1.5). COI1 acts in this system as an F-box protein in the Skp1/Cullin/F-box (SCF) to form the ubiquitination complex with SCF^{COI1} (Chini *et al.,* (2007); Thines *et al.,* (2007); Yan *et al*., (2007)). JA-Ile triggers a conformational change in the SCF^{COI1} complex, which facilitates the binding between the complex and JAZ proteins. SCF^{COI1} then ubiquitinates JAZ proteins, targeting them for proteasomal degradation (Thines *et al.,* 2007) (Fig.1.4 and Fig.1.5).

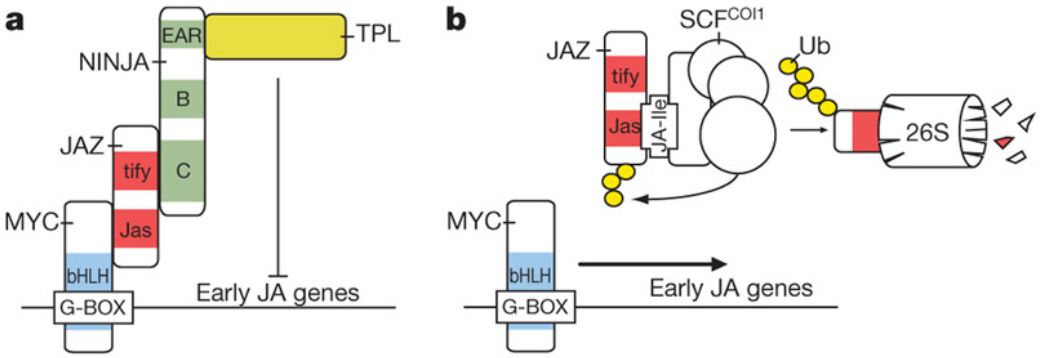


Figure 1.4 - Structure and action of JAZ repressor proteins - a, In the absence of jasmonates, basic helix–loop–helix (bHLH) MYC factors interact with the Jas domain of JAZ proteins that interact through their TIFY motif with domain C of NINJA. The EAR motif of NINJA is essential for interaction with the TPL co-repressors. b, In the presence of JA-Ile, JAZ proteins interact with the ubiquitin ligase SCF/COI1, leading to JAZ degradation in the proteasome and subsequent release of the NINJA–TPL complex from the MYC factors and activation of jasmonate-responsive genes (Reproduced with permissions from Pauwels *et al.,* 2010)

This degradation allows the targets of the JAZ proteins to be derepressed. Targets of JAZ proteins are usually TFs (for example, MYC2/3/4 (Fernández-Calvo *et al.* 2011), EIN3 and Ethylene-Insensitive3-Like 1 (EIL1) (Zhu *et al.,* 2011)). A single JAZ protein (JAZ1) is capable of binding to at least 31 proteins with a diverse range of functions (Table 1.1). Many protein targets of JAZ1 are also targets of the majority of the JAZ family (such as Jasmonate Associated Myc2 Like 2 (JAM2) and MYCs2/3/4 which interact with most JAZ proteins). When JAZ proteins are degraded TFs such as the MYCs can positive regulate the expression of genes involved in the defence response and several other pathways (Fig.1.5).

Table 1.1 – JAZ1 protein-protein interactions. Interactions observed between JAZ1 and other proteins either *in planta* or *in yeasta*. Grey – confirmed by more than one method.

ATG	Name	Reference
AT2G46510	**AIB**	Song S (2013)
AT4G00870	**AT4G00870**	Song S (2013)
AT5G14260	**AT5G14260**	Braun P (2011)
AT2G39940	**COI1**	Melotto M (2008)
AT1G22920	**CSN5A**	Braun P (2011)
AT3G20770	**EIN3**	Zhu Z (2011)
AT5G63110	**HDA6**	Zhu Z (2011)
AT3G26744	**ICE1**	Hu Y (2013)
AT1G01260	**JAM2**	Song S (2013)
AT4G16430	**JAM3**	Song S (2013)
AT5G13220	**JAZ10**	Chung HS (2009)
AT3G43440	**JAZ11**	Chung HS (2009)
AT5G20900	**JAZ12**	Pauwels L (2010)
AT1G48500	**JAZ4**	Chini A (2009)
AT1G30135	**JAZ8**	Braun P (2011)
AT1G30135	**JAZ8**	Chini A (2009)
AT1G30135	**JAZ8**	Chung HS (2009)
AT3G14080	**LSM1B**	Braun P (2011)
AT3G49580	**LSU1**	Braun P (2011)
AT5G24660	**LSU2**	Braun P (2011)
AT3G27810	**MYB21**	Song S (2011)
AT5G40350	**MYB24**	Song S (2011)
AT1G32640	**MYC2**	Melotto M (2008)
AT5G46760	**MYC3**	Niu Y (2011)
AT4G17880	**MYC4**	Niu Y (2011)
AT5G46830	**NIG1**	Qi T (2015)
AT4G28910	**NINJA**	Pauwels L (2010)
AT2G01570	**RGA1**	Hou X (2010)
AT1G12860	**SCRM2**	Hu Y (2013)
AT3G11830	**TCP-1**	Braun P (2011)
AT1G74950	**TIFY10B**	Chung HS (2009)
AT1G70700	**TIFY7**	Chini A (2009)

The most well researched interactor of the JAZ family is MYC2 (Chini *et al.* (2007); Chini *et al.* (2009)). MYC2 has been termed the 'master regulator' of the JA signalling pathway and when free of repression this TF forms homo- or hetero dimers with MYC3 and MYC4 (Fernández-calvo *et al.* (2011); Kazan and

Manners (2013); Woldemariam *et al.* (2011)). MYC complexes can then initiate the transcription of numerous JA responsive genes (Fig.1.5) (Abe *et al.* (1997); Boter *et al.* (2004); Kazan and Manners (2013); Yadav *et al.* (2005)). MYC2 can act as both a negative and positive regulator of JA pathways (extensively reviewed in Kazan and Manners 2008). This TF has been shown to be a positive regulator in the defence against insects, wound response, oxidative stress and flavonoid metabolism pathways (Dombrecht *et al.,* 2007), whereas MYC2 negatively regulates plant defence against *B. cinerea* (Zhu *et al.,* 2011).

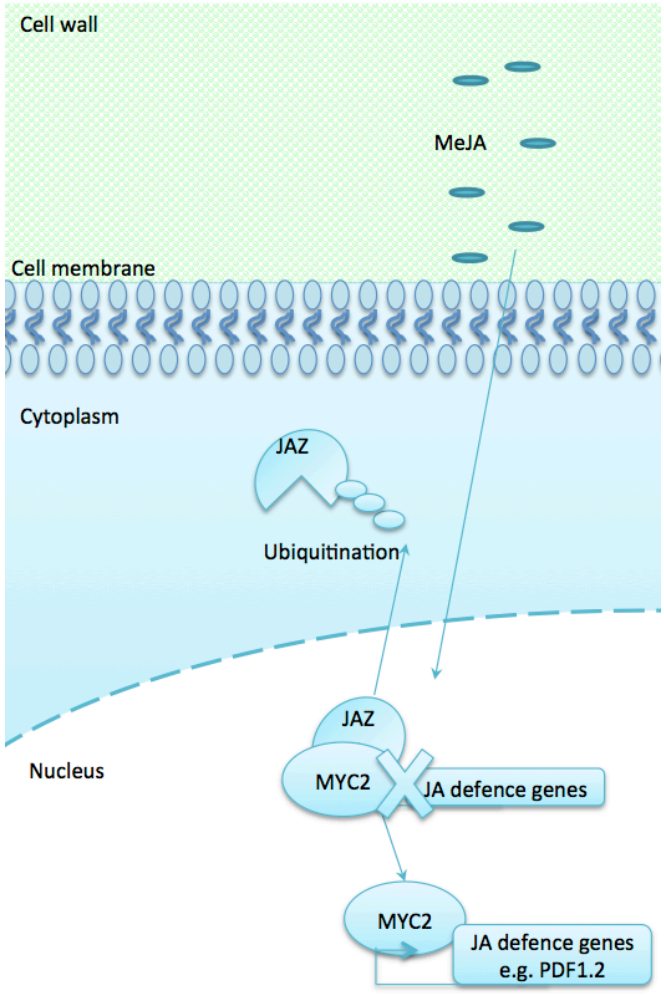


Figure 1.5 – JA signalling pathway. MeJA is converted into JA-Ile and this is perceived by COI1. COI1 forms a ubiquitination complex with SCF (SCF^{COI1}) with JA-Ile as a substrate and this complex removes JAZ proteins from their repressive positions by ubiquitination followed by proteasomal degradation. Once JAZ repression is removed proteins such as MYC2 can then regulate downstream JA responses by regulating the expression of JA responsive genes.

1.2.2.2.1 Role of jasmonic acid in *B. cinerea* defence

The previously mentioned time series transcriptomic profiling experiment performed by Windram *et al.* (2012) provided excellent insight into the role of the JA pathway in plant defence against *B. cinerea*. At 16 hpi a cluster of genes upregulated in response to *B. cinerea* inoculation were overrepresented for responses to JA, DEGs at this time included *MYB108*. *MYB108-OX* and knockout (KO) lines have shown enhanced or decreased defences against *B. cinerea*, respectively (Mengiste *et al.,* 2003). Moreover, many JA biosynthetic genes were upregulated between 12-14 hpi (Windram *et al.,* 2012). As mentioned previously, when the JA receptor (COI1) is mutated plants are more susceptible to *B. cinerea* (Thomma *et al.,* 1998). Since MYC2 negatively regulates *myc2-2* was more resistant to *B. cinerea* than WT (Zhu *et al.,* 2011).

EIN3 appears to be a convergence point for the JA and ET pathways and likely accounts for the synergy observed between the two pathways. However, how the pathways converged to control EIN3 expression was for a long time the topic of wide speculation until it was found that this is likely facilitated by the JAZ proteins (Zhu *et al.,* 2011) (Fig.1.6).

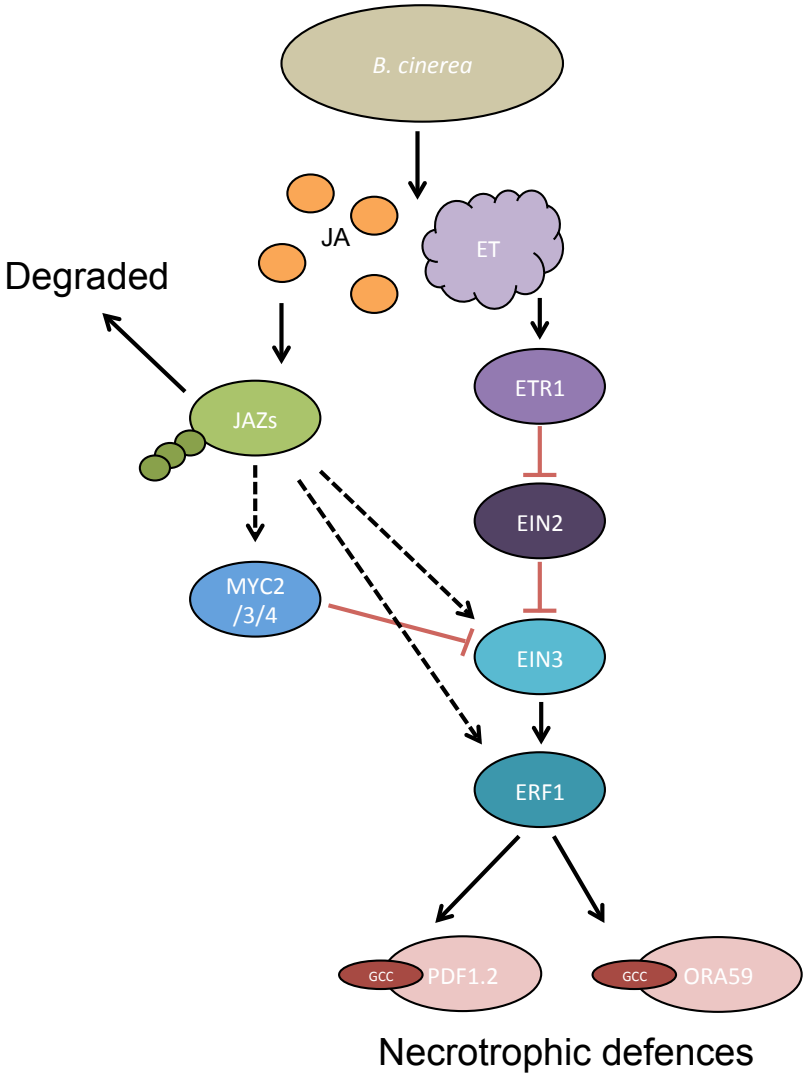


Figure 1.6 – JAZs mediate cross talk between ET/JA pathways in response to *B. cinerea* attack. Under uninfected conditions JAZs repress the action of EIN3, MYC2/3/4 and ERF1 (A). Upon the perception of *B. cinerea* JA and ET are released, relieving JAZs of their repressive roles allowing MYC2/3/4, EIN3, ERF1 to be activated and in turn downstream defences to be activated by ERF1 binding the GCC box in promoter regions. Repression indicated by red lines. Solid black lines indicate positive regulation. Dashed black lines in B show genes/proteins that are no longer repressed by JAZ proteins. (Information for figure from Song *et al.,* 2014).

1.2.2.3 Salicylic acid

SA is crucial to plant defences against a wide range of pathogens (as reviewed in Vlot *et al.,* 2009). However, despite the prominent role SA plays in PTI and ETI the molecular dynamics of SA signalling are largely unknown, so much so that up until 2012 the SA receptor was unidentified.

The NPR1 (non-expresser of PR genes) protein is a central component of the plant defence response and plants lacking in NPR1 are susceptible to numerous biotrophic and hemi-biotrophic pathogens (Cao *et al.* (1994); Delaney *et al.* (1995)). NPR1 directly binds SA and had been speculated to be the SA receptor (Wu *et al.,* 2012). However, Fu *et al.,* (2012) found no interaction between NPR1 and SA, but did discover that SA interacted with NPR3/4 and from this finding it was postulated NPR3/4 were SA receptors. Given this conflicting evidence Manohar *et al.,* (2015) explored the binding affinity of SA to NPR1 and found that NPR1 is indeed bound by SA and is likely an SA receptor. It was also found that SA binds nine other plant proteins, however, the implications of these interactions needs to be further investigated (Manohar *et al.,* 2015).

Under conditions of low SA NPR1 is localised to the cytoplasm as an oligomer, however, when SA levels rise NPR1 converts from an oligomer to a monomer and enters the nucleus (Caarls *et al.,* 2015). Upon nuclear relocalisation NPR1 binds TGACG sequence-specific binding protein (TGA) TFs. The newly formed TGA/NRP1 complex then activates SA responsive genes including *PATHOGENESIS RELATED 1 (PR1)* (Caarls *et al.,* 2015). *PR1* is a marker gene for SA activity and PR genes have been shown to encode antimicrobial proteins (Stintzi *et al.* (1993); Cao *et al.* (1994), Durrant and Dong, (2004)). Under low SA levels, *PR1* expression is repressed by the TF, TGA2 (Zhang *et al.,* 2003) and in response to increased SA NPR1 interacts with TGA2 to form an enhancer, which binds to the *PR1* promoter and positively regulates *PR1* expression (Boyle *et al.,* 2009). This binding is mediated via the TGA binding motif found upstream of numerous SA mediated genes. As well as activating expression of SA responsive genes the TGA TFs are also responsible for the repression of several JA responsive genes (Leon-Reyes *et al.,* 2010).

1.2.2.3.1 Role of salicylic acid in *B. cinerea* defence

SA is involved in the defence response against *B. cinerea* given the repressive effect SA has on the JA and ET signalling pathways (see 1.2.2.5.1). SA suppresses

the JA/ET signalling pathway by repressing the action of the ORA59 TF (Van der Does *et al.,* 2013) (Fig.1.2). In doing so SA reduces the expression of many genes in the JA/ET signalling pathways that contain the GCC binding motif in their promoter regions (Van der Does *et al.,* 2013).

However, host defence against *B. cinerea* is more complex than just the antagonism between SA and JA/ET. For example, TGA3, an interactor of NPR1 (Kesarwani *et al.,* 2007), has been shown to be significantly transcriptionally downregulated 18 hpi following *B. cinerea* inoculation (Windram *et al.,* 2012). Two independent knockout lines of *TGA3* showed increased susceptibility *to B. cinerea* inoculation showing *TGA3* to have a role in positively regulating host defence against *B. cinerea* (Windram *et al.,* 2012).

1.2.2.4 Plant defence involves more hormones than SA, ET and JA

While SA, JA and ET appear to be the key hormones involved in the plant defence pathways, other hormones with prominent roles in development also play pivotal roles in the defence response (extensively reviewed in Robert-Seilaniantz *et al.,* 2011). The role of ABA has been discussed above in section 1.1.4.

Auxin, has a role in plant defences against biotrophic pathogens, although again its role is not fully understood. Nonetheless, repression of auxin signalling has been shown to increase susceptibility to the necrotrophic pathogens; *B. cinerea*, *Plectosphaerella cucumerina* (Llorente *et al.,* 2008) and *A. brassicicola* (Qi *et al.,* 2012). This defence role of auxin appears to be regulated by miRNAs. For example, *miR393* transcription is induced in response to *flg22* treatment, *miR393* then targets three auxin receptor mRNA transcripts, which reduces auxin induced gene expression and increases resistance to *P. syringae* (Navarro *et al.,* 2006).

Gibberellic acid (GA) signalling has similar mechanisms to what has been outlined for JA signalling, with DELLA proteins acting in a comparable way to

JAZ proteins to repress the GA signalling pathway by repressing Phytochrome Interacting Factor (PIF) TFs (Robert-Seilaniantz *et al.,* 2011). DELLA proteins play key roles in defence, mutations in these proteins lead to increased resistance against *P. syringae* (Navarro *et al.,* 2006). DELLA proteins activate a pathway involved in the positive induction of genes related to ROS detoxification (Achard *et al.,* 2008). Therefore, the mutation of a gene encoding for a DELLA protein, RGL3, unsurprisingly increases susceptibility to *B. cinerea* given that a virulence mechanism of this necrotroph is the release and generation of ROS induced stress (Jacobs *et al.,* 2011). It has been suggested that the physical interaction between DELLA proteins and JAZ proteins contribute to the positive influence ABA has on the defence response- JAZ proteins are bound by DELLA proteins preventing the repressive action they have on TFs such as MYC2, hence allowing the JA signalling pathway to remain active (Hou *et al.,* 2010).

1.2.2.5 Cross-talk

The roles of hormones such as ABA, auxin and GA in pathogen defence are unclear in pathogen defence; it appears the main role of these hormones is may be in modulating the interplay between JA and SA (Pieterse *et al.,* 2012).

1.2.2.5.1 Interplay between JA and SA

As discussed, both JA and SA have key roles in plant defence against pathogens, JA is the key hormone involved in defence against necrotrophic pathogens whereas SA plays a prominent role in defence against biotrophic and hemi-biotrophic pathogens (Glazebrook, 2005). Given the opposing lifestyles of these pathogen groupings, the hormonal pathways that defend against them are generally considered antagonistic to one another (Bostock (2005); Beckers and Spoel (2006); Niki *et al.* (1998)). However, synergy between the JA and SA pathways has also been observed but this observation is uncommon (Glazebrook (2005); Kim *et al.,* (2014)).

Examples of JA antagonizing the SA defence pathway are numerous; Arabidopsis plants mutated in JA signalling genes *MPK4*, *SUPPRESSOR OF SA*

INSENSITIVITY 2 (SSI2) or *COI1* show a reduction in JA signalling as well as constitutive expression of SA marker genes (Kachroo *et al.* (2001); Kloek *et al.* (2001); Petersen *et al.* (2000)). This increased SA activity enhanced plant defence against both *P. syringae* and *P. parasitica* (Kachroo *et al.* (2001); Kloek *et al.* (2001); Petersen *et al.* (2000); Shah *et al.* (2001)).

Examples of SA repressing the JA defence pathway are also abundant, plants mutated in *PHYTOALEXIN DEFICIENT 4 (PAD4)* and *ENHANCED DISEASE SUSCEPTIBILITY 4 (EDS4*) which regulate SA biosynthesis showed decreased SA levels (Gupta *et al.,* 2000). Both *pad4* and *eds4* mutants also showed increased JA signalling when treated with JA signalling elicitors; this was based on a measurement of *PDF1.2* (a JA signalling marker gene) expression (Gupta *et al.,* 2000).

How this cross talk is achieved is not yet fully understood, however it has been predominantly attributed to transcriptional regulation (Van der Does *et al.,* 2013), other mechanisms behind this cross talk are extensively reviewed in Caarls *et al.,* (2015).

1.2.2.5.2 Cross-talk between the ET and SA/JA pathways

ET acts synergistically with JA as a positive regulator of the defence response against necrotrophic pathogens and appears to act as a negative regulator in the defence response against biotrophic pathogens (Glazebrook, 2005). Fittingly, *ERF1-overexpressor (OX)* mutants show increased resistance to the necrotroph *B. cinerea* and reduced resistance to the hemi-biotrophic pathogen *P. syringae* (Berrocal-Lobo *et al.* (2002); Berrocal-Lobo *et al.* (2004)).

Examples of ET and JA synergy in the necrotrophic defence response pathways are abundant; defence related genes, such as *PDF1.2,* are positively transcriptionally regulated by both ET and JA (Penninckx *et al.,* 1998). EIN3 has an integral regulatory role in ET signalling, similar to MYC2 in JA signalling. EIN3 not only regulates the ET signalling pathway but also has a functional role in the JA signalling pathway (Song *et al.,* 2014). EIN3 is repressed by MYC2 and

it has been hypothesized this MYC2-EIN3 interaction is the key between balancing several JA signalling pathways. EIN3/EIL1 positively regulate necrotrophic defences and root hair development, whereas MYC2/3/4 regulate wound responsive genes, herbivore defences and metabolism (Song *et al.,* (2014); Zhu *et al.,* (2011)). MYC2 inhibits EIN3/EIL1 action by protein binding and so *ERF1* transcription is not activated, therefore key genes involved in necrotrophic defences are not transcribed and the defence response is repressed. More research is needed fully to understand this interaction, however, Song *et al.,* (2014) eluded to the possibility that the JAZ proteins could be playing a crucial role in fine tuning the MYC2 and EIN3 interactions. JAZ1, JAZ3 and JAZ9 have all been shown to be able to bind EIN3 at the protein level (Zhu *et al.,* 2011).

Although JA and ET are generally considered synergistic, modeling data has previously shown ET to inhibit the JA pathway (Kim *et al.,* 2014). Data on ET and SA interactions are limited. However, the previously mentioned NPR1 dependent SA mediated JA repression appears to be mediated by ET, with ET modulating the positive or negative action of NPR1 (Leon-Reyes *et al.,* 2009).

1.2.2.5.3 Cross-talk between other hormones and the SA/JA pathways

ABA signalling plays a crucial role in mediating plant defences. ABA has been shown to suppress plant immunity by antagonizing SA related defences (Cao *et al.* (2011); De Torres-Zabala *et al*. (2009); Jiang *et al.* (2010); Yasuda *et al*. (2008)). ABA has been shown to act in synergy with JA pathways (Abe *et al*. (2003); Anderson *et al*. (2004)). ABA represses the EIN3 branch of JA immunity and enhances the MYC2 regulated pathway of the JA responses (Abe *et al*. (2003); Anderson *et al*. (2004)). By enhancing this pathway ABA appears to increase responses to herbivory and wounding and suppress host immune responses to necrotrophic pathogens (Fernandez-Calvo *et al.* 2011).

Auxin acts antagonistically with SA, it suppresses both SA signalling and synthesis (Navarro *et al.* (2006); Robert-Seilaniantz *et al.* (2011)). When auxin levels are suppressed and hence SA levels are higher, resistance to both *P. syringae* and *H. arabidopsidis* is increased and defences against the necrotrophic

pathogen *A. brassicicola* is decreased (Robert-Seilaniantz *et al.,* 2011). This is likely due to the cross talk between SA and JA.

GA is a positive mediator of plant growth. GA signalling repressor proteins, DELLA proteins, have roles in the plant immune response and the JA signalling pathway. DELLA interactions with the GA and JA signalling pathways are thought to fine-tune the plant balance between growth and defence responses. *della* mutants show decreased JA responses and *DELLA-OX* plants show enhanced JA responses (Navarro *et al.,* 2008). DELLA proteins mediate the cross talk between JA and GA signalling by binding to the JAZ proteins and preventing their repression of TFs such as MYC2/3/4 hence increasing the JA signalling pathway in times of the low GA (Hou *et al*., (2010); Yang *et al.,* (2012)).

Under conditions of low red:far red (R:FR) light plants reduce JA defence related signalling and increase plant growth by increasing the degradation of DELLA proteins. Reducing DELLA levels in turn increases JAZ activity, in particular the activity of JAZ10, however other JAZ proteins have not yet been tested (Leone *et al*., 2014).

In summary, it appears phytohormone signalling and cross-talk between phytohormone signalling pathways are crucial to the plant defence response against a wide variety of pathogens.

1.3 Transcriptional reprogramming in plant defence

In eukaryotic organisms gene expression is tightly controlled by an array of regulatory mechanisms. One of which is DNA packaging- to fit the entire genome into a nucleus DNA is tightly packaged around histones. This packaged DNA is referred to as chromatin. As well as acting as a mechanism to fit all DNA into the nucleus, the packaging of chromatin also acts to regulate the accessibility of DNA to transcription. In general, only when chromatin is accessible or 'open', can the genes within the chromatin be transcribed and expressed.

Even in areas of open chromatin where genes are accessible transcription will not occur unless the correct regulatory machinery is in place. In general, in the upstream promoter region of a gene a TATA box will be present, which is bound by an RNA polymerase II (RNA Pol). The binding and holding of the polymerase in place is facilitated by an array of TFs- this complex is known as the transcription-initiation complex. TFs bind directly to specific regions of the promoter known as *cis*-regulatory regions. Other regulatory sequences within the genome are enhancers and silencers, where binding of activators and repressors, respectively, occurs. During transcription the enhancer/silencer containing chromatin undergoes a conformational change and is 'looped' so that these sequences are parallel to the transcription-initiation complex. Activators or repressors then bind to the target sequences and interact with the transcription-initiation complex to either initiate or repress transcription of the gene.

Transcriptional regulation in eukaryotes is a complex non-linear process (Heinrich and Schuster (1996); Mazur *et al.* (2009)). TFs modulate this process and are often key regulators of gene expression networks. Transcriptional reprogramming is a major part of the host defence response against pathogens (comprehensively reviewed in Tsuda and Somssich, 2015). This reprogramming occurs in response to signals from immune receptors such as the chitin receptor, CERK1, which activate TFs, and these TFs then form multi-protein complexes to regulate gene expression (Moore *et al.,* 2011). An example of such transcriptional reprogramming in the immune response was outlined above in relation to MPK4/MSK1 regulation of WRKY33 and *PAD3* during *B. cinerea* infection.

The importance of host transcriptional reprogramming in response to *B. cinerea* infection is extremely significant as evidenced by numerous transcriptome profiling studies (AbuQamar *et al.* (2006); Ferrari *et al.* (2007); Rowe *et al.* (2010); Mulema and Denby (2011); Birkenbihl *et al.* (2012); Windram *et al.,* (2012)). Significant transcriptional reprogramming occurs in plant cells within

48 hours of infection with *B. cinerea*, with approximately one third of the Arabidopsis genome differentially expressed (DE) (Windram *et al.,* 2012).

Windram *et al.,* (2012) demonstrated that transcriptomic changes were tightly correlated with *B. cinerea* growth stages. Initially, *B. cinerea* undergoes a rapid growth stage between 4-12 hpi, a short lag phase is then observed in fungal growth between 12-16 hpi, the fungus then resumes rapid growth between 16-20 hpi. By 20-28 hpi the fungus enters a significant lag period in growth. These fungal growth stages are reflected in host transcriptional responses (Fig.1.3). The initial lag phase observed in *B. cinerea* growth coincides with the upregulation of DEGs involved in several defence related process suggesting that the transcriptional response of the plant temporarily impedes fungal growth. The second lag phase in fungal growth (20-28 hpi) is when lesion formation occurs and this coincides with a transcriptional shift in the host. Both occurrences in lag phases coincides with peaks in the number of host DEGs suggesting significant interaction between host and pathogen.

Several other studies have identified thousands of Arabidopsis transcripts that change in expression following *B. cinerea* infection (Ferrari *et al.,* 2007; Rowe *et al.,* 2010; Birkenbihl *et al.,* 2012; Mulema and Denby, 2012), indicating a key role for TFs in synchronizing these changes. In addition, many studies have employed functional analysis of altered expression mutants to identify numerous TFs involved in defense against *B. cinerea.* TFs involved in the host response to *B. cinerea* include ERF1 (Berrocal-Lobo *et al.,* 2002), WRKY33 (Birkenbihl *et al.,* 2012), WRKY70 (AbuQamar *et al.,* 2006), PAD2 (Ferrari *et al.,* 2003), PAD3 (Ferrari *et al.,* 2007), ERF9 (Maruyama *et al.,* 2013), Botrytis-Susceptible 1 (BOS1), (Mengiste *et al.,* 2003), ERF5/6 (Moffat *et al.,* 2012), TGA3 (Windram *et al.,* 2012), ORA59 (Pré *et al.,* 2008), EIN3/ERF1 (Solano *et al.,* 1998), Nac Domain Protein 1 (NAC1) (Wang *et al.,* 2009) amongst others. The roles of ORA59, WRKY33 and EIN3 are highlighted in Fig.1.2. Further details on TFs in phytohormone pathways are outlined below.

1.3.1 Beyond transcriptomic profiling

B. cinerea receptors in the cell have been identified (Fig.1.2) and the high-resolution time-series from Windram *et al.,* (2012) gave an exceptional level of detail on the transcriptomic changes that occur in a plant cells in response to *B. cinerea* inoculation. However, there is a large knowledge gap as to how perception by receptors is linked to transcriptional changes (Ma *et al.,* 2011). For example, apart from the link between MPK1 and WRKY33 very little is known about how the MAPK cascade mechanistically links to transcriptional changes within the cell.

As discussed above, chromatin accessibility has been associated with the plant immune response. Posttranslational modifications of chromatin structure often occur via histone modification, these modifications alter the accessibility of chromatin, which can directly alter the expression of genes in those regions of chromatin. Accessibility of chromatin can be altered by modification to histone tails through acetylation, methylation, phosphorylation, ubiquitination, sumoylation, carbonylation or glycosylation. Histone modifications are facilitated by various protein groups, including histone acetyltransferases (HATs) or histone deacetylases (HDACs). HATs acetylate histones, which is associated with increased transcriptional activation and HDACs deacetylase histones, which is associated with transcriptional suppression. Chromatin remodeling enzymes (CMEs) do not alter histone tails; instead CMEs adjust interactions between DNA and histones to assist in the relocation or dissociation of nucleosomal structures.

Several HDACs have been associated with differential plant immunity to pathogens; in rice the HDAC HDT701 increases resistance to the rice blast fungus by upregulating *MAPK6* transcription (Ding *et al.,* 2012). CMEs have also been implicated in the plant defence response. The CME, *SPLAYED (SYD)* increases susceptibility to *B. cinerea* by reducing the expression of ET/JA responsive genes such as *PDF1.2* (Walley *et al.,* 2008).

1.3.1.2 ATAC-Seq identifies active regulatory regions

The function of each gene is controlled by several *cis*-regulatory elements. Although the extensive transcriptomic time series data by Windram *et al.,* (2012) allowed for regulatory interactions to be inferred using network modeling techniques, these interactions still need to be confirmed *in planta*. The network modeling allowed for the prediction of regulatory relationships between TFs and downstream gene expression changes. A regulatory gene network of predicted interactions was formed. Within the network several TFs were predicted to be 'hubs', that is TFs that are regulating the expression of multiple genes involved in *B. cinerea* defence. Screening plant mutants with altered expression of these hub genes it has been observed that TFs deemed to be 'hubs' were significantly more likely to change the plant resistance level against *B. cinerea* when mutated in expression than if a non-hub gene was altered in expression and mutant plants screened (PRESTA group, unpublished). To confirm this regulatory model of the gene interactions that occur during *B. cinerea* infection, it needs to be elucidated where the 'hub' TFs are binding and how this binding is influencing gene expression.

Techniques, such as chromatin immunoprecipitation (ChIP), can investigate these regulatory relationships, but such techniques have struggled to capture the full genome. ChIP-seq and ChIP-chip are both powerful approaches to map all the binding sites of one specific protein in the full genome. Mapping the binding sites of the 'hub' TFs using this technique would be an expensive and laborious task. Yeast-1hybrid (Y1H) is another technique to identify TFs that are binding to specific regions of DNA. However, this technique is performed *in-yeasta* and so may not reflect what is binding *in-planta*, just what is capable of binding. Again, mapping the interactions of all 'hub' TFs with all DNA regions would be an onerous task, although an extremely informative one. Although the 'hub' TFs are of interest it would be most informative to identify interactions between not only the hub TFs but also interactions between downstream TFs and genes, therefore a genome-wide approach would be most beneficial to

explore the regulatory interactions governing the plant defence response against *B. cinerea*.

Current genome-wide techniques that would identify all cis-regulatory regions include DNase I hypersensitive sites sequencing (DNase-seq), Formaldehyde-Assisted Isolation of Regulatory Elements sequencing (FAIRE-seq) and Assay for Transposase-Accessible Chromatin coupled with high throughput sequencing (ATAC-Seq). FAIRE-seq requires a very high cell number and gives low signal to noise ratios making computational analysis difficult and is therefore unlikely to give the high-resolution data required to identify TF occupancy at a single nucleotide level (Tsompana and Buck, 2014).

DNase-Seq makes use of the DNase I enzyme which cuts DNA, all chromatin protected by nucleosomes or TFs will be protected from the enzyme and therefore less cuts will occur in those regions (Song *et al.,* 2010). This allows for a high resolution mapping of open chromatin and can identify regions of TF occupancy (Song *et al.,* 2010). However, due to the enzymatic reaction within this protocol it can be very difficult to optimize (Tsompana and Buck, 2014).

When compared with DNase-Seq, ATAC-Seq requires a shorter laboratory protocol (meaning transient interactions between DNA and proteins are more likely to be captured) and a reduced cell number is required, however, the data obtained is of similar sensitivity and accuracy (an extensive review of the advantages was carried out by Tsompana and Buck, 2014).

ATAC-Seq makes use of a hyper-activated Tn5 transposase which both fragments and tags areas of open chromatin (tagmentation); these tagged areas are then amplified and then undergo paired-end sequencing. The transposase can only insert itself and fragment areas of open chromatin hence why peaks of short reads are seen in regions of open chromatin. Moreover, this fragmentation can reveal chromatin occupied by TFs as these regions have a distinctive fragmentation footprint.

Due to the numerous advantages of ATAC-Seq over other genome-wide mapping techniques, this was selected to identify genome wide TF occupancy in Chapter 5. This data will reveal differences in TF occupancy at different times of day when differential resistance to *B. cinerea* has been observed.

1.4 Plant circadian clock

Disrupting components of the plant defence response has obvious effects on the susceptibility of the plant to pathogens. This has been strongly evidenced by numerous gene knockouts altering plant immunity against a wide variety of pathogens. Perhaps more unexpectedly, however, is the recently described impact of the circadian clock on the susceptibility of plants to pathogens, whereby the time of day when the plant is infected significantly influences the outcome of that infection event (Bhardwaj *et al.,* (2011); Ingle *et al.,* (2015); Wang *et al.,* (2011); Zhang *et al.,* (2013b)).

1.4.1 General overview

The circadian clock enables organisms to anticipate recurrent environmental events such as sunset and sunrise as well as allowing distribution and synchronisation of behaviours to optimal times throughout the day (Nagano *et al.,* 2012).

Circadian clocks are conserved across a wide range of species, from plants to animals to fungi (Young and Kay, 2011). Most circadian clocks have been shown to receive entrainment from environmental changes in light and dark or/and hot and cold conditions that cycle rhythmically over an approximately 24 h day (Fig.1.7). The central oscillator of the circadian clock in many organisms shares many similarities notably the entailing of several interlocking positive and negative transcription and translation feedback loops (TTFL) between the core circadian clock genes, which can then confer rhythmicity to the rest of the genome (Fig.1.7). It is generally agreed that the clock can be simplified to a three-tier system which, firstly, receives input from the environment, secondly,

integrates this with the central oscillator that then finally modulates a number of downstream effects (Fig.1.7).

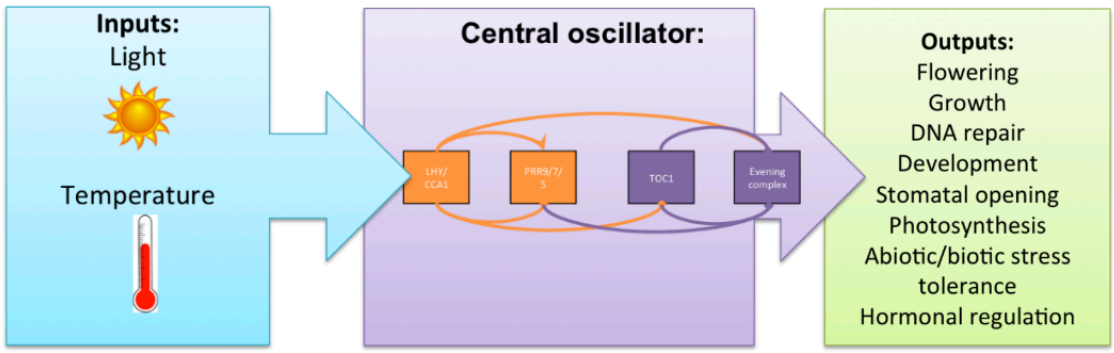


Figure 1.7 - Input/output model of the Arabidopsis circadian clock - The central oscillator in *A. thaliana* is entrained by light and temperature cycles that occur daily. This then results in rhythmicity in much of the genome. The central oscillator is comprised of three main genes; *LHY* (*LATE ELONGATED HYPOCOTYL), CCA1 (CIRCADIAN CLOCK ASSOCIATED 1*) and *TOC1* (*TIMING OF CAB EXPRESSION 1*) along with the PRR genes (*PRR5, PRR7, PRR9*). *LHY* and *CCA1* are expressed at dawn and act redundantly to repress *TOC1* and *PRR5* (Carré *et al.,* 2013). *TOC1* then acts alongside other clock components (*PRR5, 7, 9*) to repress *CCA1/LHY* expression during the day (Carré *et al.,* 2013). The Evening Complex (EC) (comprising of the proteins; ELF3, ELF4 and LUX) then works during the night and represses *TOC1, PRR7* and *PRR9* expression thus allowing the gradual increase of *CCA1/LHY* levels at dawn (Carré *et al.,* 2013). Purple lines within the central oscillator model – repressive influence of evening complex, orange lines – repressive influence of the morning complex.

1.4.2 Central oscillator

The current model of the main circadian oscillator in Arabidopsis is rapidly changing (Carre and Vefingstad (2013); Fogelmark *et al.,* (2014); Gendron *et al.,* (2012); Greenham and McClung *et al.,* (2015); Nakamichi *et al.,* (2007); Pokhilko *et al.,* (2012)). Current understanding is that the circadian central oscillator in Arabidopsis is composed of two halves - those genes expressed at dawn (*CCA1/LHY, PRR9/7/5*), and those expressed in the evening (*TOC1, LUX, ELF3/4*) (Lai *et al*. (2012); Nakamichi *et al.* (2012)) (Fig.1.8). These genes then interact with one another via positive or negative TTFL to create an endogenous mechanism that enables the plant to anticipate and respond to external stimuli (Anwer *et al.,* 2013) (Fig.1.8).

CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) are expressed simultaneously with expression peaks approximately two hours after dawn (Carre and Vefingstad, 2013). Following this, *PSEUDO-RESPONSE-REGULATORS* (*PRRs*) transcript levels peak sequentially throughout the day; *PRR9* expression peaks in the early morning followed by *PRR7, 5* and *TOC1* (Nakamichi *et al.,* 2012). *TOC1* expression at the end of the day correlates with a peak in *LUX ARRHYTHMO* (*LUX*) and *EARLY FLOWERING 3 and 4* (*ELF3/4*) expression. *LUX* and *ELF3/4* proteins form the evening complex (EC) (Fig.1.8) (Carre & Vefingstad, 2013). The strict regulation of the clock gene expression is maintained by a central feedback loop (Fig.1.8). As well as the TTFL, Arabidopsis has been shown to have circadian redox rhythms that influence the TTFL in the central oscillator (Edgar *et al.,* 2012).

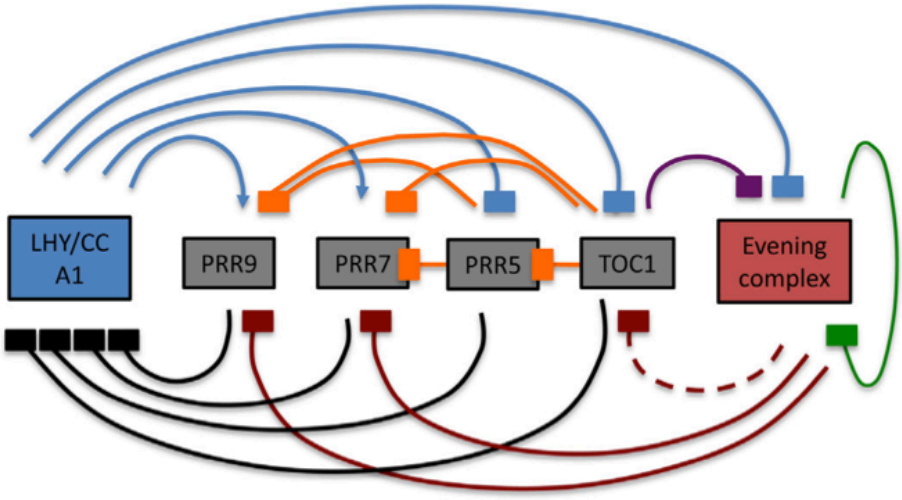


Figure 1.8 – Arabidopsis circadian clock transcriptional model. Blue arrows – *LHY* and *CCA1* are simultaneously expressed at dawn to repress *TOC1* & *PRR5* and activate *PRRs 7* & *9*. Grey arrows – Then throughout the day *TOC1* and *PRRs 9, 7* & *5*, inhibit *LHY* and *CCA1*. Red arrows – *PRR/TOC1* expression is then stopped at night by *ELFs 3* & *4* and *LUX* (acting in the evening complex), which allows *LHY/CCA1* to be transcribed at dawn. Dashed line – may be an indirect effect. Orange arrows – Later members in the PRR cascade repress earlier PRRs potentially allowing for temporal expression. Green arrow-Members of the evening complex are in an auto regulatory loop only allowing for transient expression. Purple arrow – *TOC1* represses the EC action. (Reproduced with permissions from Carre and Veflingstad, 2013)

1.4.3 The circadian clock has many roles in Arabidopsis

A strong circadian clock confers an adaptive advantage to Arabidopsis plants (Dodd *et al.,* 2005), likely due to the widespread regulation that it has on the Arabidopsis genome. The clock confers this rhythmicity to the genome using both transcriptional and post-transcriptional mechanisms.

Early transcriptional analysis revealed that the circadian clock controls 10-15% of the Arabidopsis genome (Harmer *et al.,* 2000) whilst a recent meta analysis of multiple microarrays has shown that up to 80% of the genome displays rhythmicity under light 12 h:dark 12 h (LD) and/or hot 12 h:cold12 h (HC) conditions. As much as 30-40% of these genes maintain a rhythmic expression pattern under constant conditions lacking in HC or LD cycles (Covington *et al.,* 2008). Direct transcriptional regulation by the clock has largely been inferred by ChIP-Seq data. TOC1 was found to be a repressor of morning phase genes with approximately 40% of all genes known to be under circadian regulation bound by TOC1 (Huang *et al.,* 2012). Moreover, ChIP-Seq of PRR5 has demonstrated that this TF is bound upstream of morning-phased genes (in agreement with its hypothesised role to repress morning phased genes (Fig.1.8)) (Nakamichi *et al.,* 2012). In contrast, PRR7 has been shown to bind to promoters of genes involved in late night or early morning processes (Liu *et al.,* 2013a). A morning component of the central oscillator (CCA1) has more recently had its binding partners revealed using ChIP-Seq, CCA1 was shown to bind to and repress genes from being expressed on a morning, these genes are then released from repression and are expressed on an evening (Kamioka *et al.,* 2016). Furthermore, a *cca1/lhy* mutant showed members of this gene set to be upregulated in the morning, indicating a functional interaction between CCA1 and this gene set (Kamioka *et al.,* 2016).

Post-transcriptional mechanisms are also employed by the clock to regulate plant processes (extensively reviewed in Romanowski and Yanovsky *et al.,* 2015). Pre-mRNA processing forms mature mRNA from premature mRNA (pmRNA) by splicing out introns and ligating the exons together. The splicing and ligating process can occur in different ways to form a variation of mRNAs

(and eventually proteins) from one pmRNA transcript, this process is known as alternative splicing. Two isoforms of CCA1 have been shown to regulate cold response genes differently (Seo *et al.,* 2012). Moreover, long non-coding RNAs (lncRNAs) are also thought to play a role in circadian regulation, as several clock genes have been found to be the antisense targets of lncRNAs (Jouannet and Crespi, 2011).

Once clock proteins have been translated, there are further regulatory steps involving protein modification, which are essential for the clocks regulation of the genome. For example, there are many kinase and phosphatase genes that are under circadian regulation (Kusakina and Dodd, 2012). Moreover, several clock genes (*TOC1, PRRs* and *CCA1*) have been shown to be phosphorylated differently at different times of day, suggesting that there is further fine tuning of the circadian oscillator at a protein level (Fujiwara *et al.* (2008); Portolés and Más (2010)).

After phosphorylation of the clock proteins, they can go on to form protein-protein interactions as yet another form of regulation. For example, LHY and CCA1 form homo and heterodimers in the nucleus where they then use the binding domains of other TFs (such as Far-Red Elongated Hypocotyls 3 (FHY3)) to bind and repress the expression of many genes (Li *et al.,* 2011).

All these mechanisms of regulation have been shown to mediate many processes throughout the plant (Fig.1.9) and are extensively reviewed in Greenham and McClung *et al.,* (2015). The following sections will give a brief outline of some of these processes.

The clock is involved in the regulation of photoperiodic flowering. Plants often flower in the summer when conditions, particularly daylength and temperature, are more favourable, and consequently plants have developed the ability to measure day length (Andrés and Coupland, 2012). Such measurements are dependent upon the circadian clock; mutations in the central oscillator genes alter flowering timing (Song *et al.,* 2015). The circadian clock has also been shown to modulate starch usage in plants (Graf *et al.,* 2010). The circadian clock

regulates the rate at which starch is degraded during the night, ensuring that there is sufficient to last the full night preventing starch starvation, which inhibits growth, but also allowing the plant to utilise starch to continue growth and respiration at night.

Plant growth is widely regulated by the clock and hypocotyl elongation is one of the most widely studied circadian processes in plants. Rhythmic elongation of hypocotyl length is observed under free-running conditions (Dowson-Day and Millar, 1999). This rhythmic elongation requires the regulation of *PHYTOCHROME INTERACTING FACTOR 4 and 5 (PIF4/5)*, which combine signals from many sources including light, sucrose levels and the circadian clock to positive mediate plant growth (Feng *et al.* (2008); Stewart *et al.* (2011)). The clock regulates *PIF4/5* expression via ELF3, a component of the EC (Nieto *et al.,* 2015).

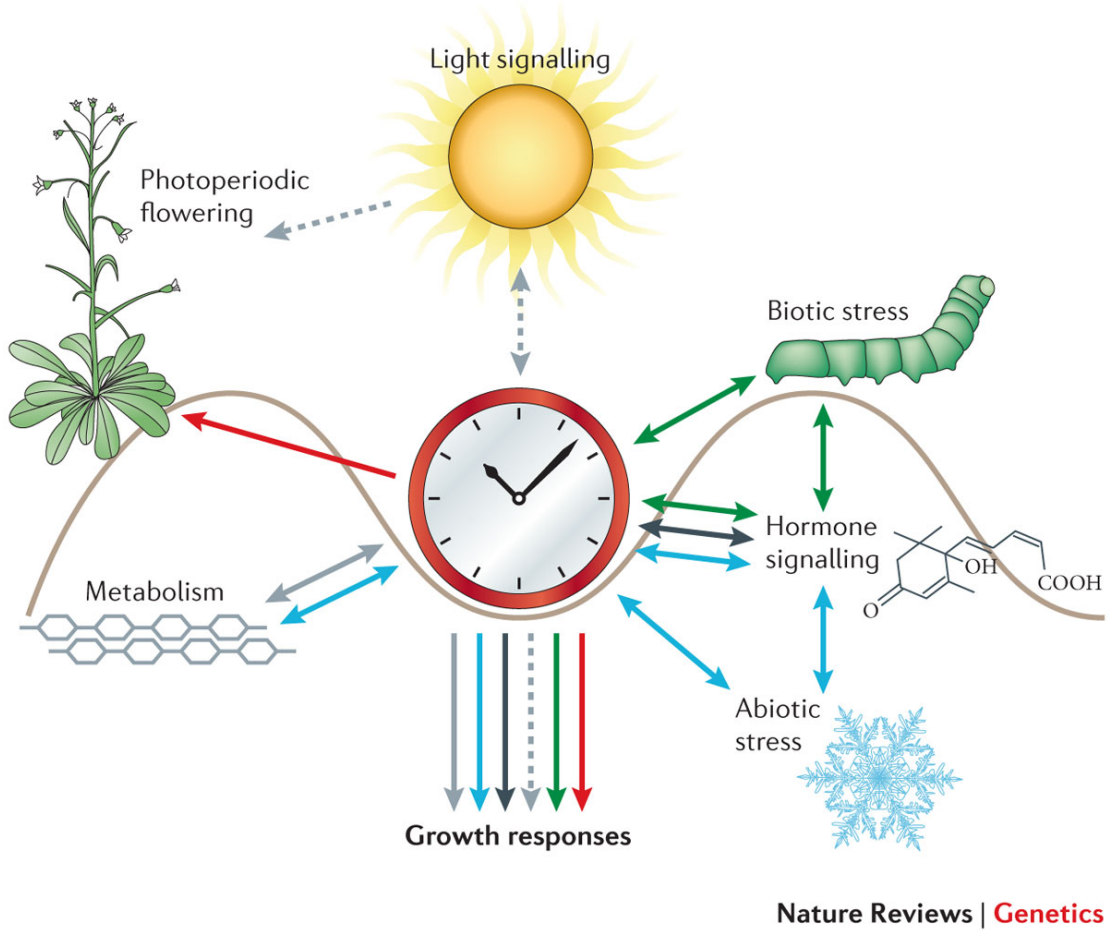


Figure 1.9 – The circadian system of plants. The circadian clock occupies a central position in the regulatory network of plants. The traditional view has been of a linear pathway in which environmental stimuli, such as light and temperature, entrain the clock, which then regulates output pathways. However, extensive data have accumulated in support of a more complex networked view in which the clock regulates its own sensitivity to entraining stimuli, for example through regulated expression of photoreceptors. Metabolic pathways, as well as abiotic- and biotic-stress responses, often show circadian regulation, but metabolic intermediates, hormones and stress-signalling pathways feed back to modulate clock function (indicated by bidirectional arrows). In addition, there is considerable crosstalk among clock-modulated signalling pathways (indicated by coloured arrows for each output pathway). Thus, the circadian clock has a central role in an integrated system to temporally coordinate physiological and metabolic responses with the environment (Reproduced with permission from Greenham and McClung *et al.,* 2015).

1.4.3.1 The circadian clock mediates abiotic stress tolerance

The clock has also been widely implicated in abiotic stress tolerance (reviewed in Grundy and Stoker *et al.,* (2015)). Several mutants of the circadian clock genes have demonstrated perturbed responses to abiotic stresses. A *prr5/7/9* mutant displayed increased tolerance to salinity, drought and freezing (Nakamichi *et al.,* 2009). A *toc1* mutant also displayed increased freezing tolerance (Keily *et al.,* 2013). Furthermore, a *toc1 RNAi* line with reduced *TOC1* levels showed an increased drought tolerance (Legnaioli *et al.,* 2009). However, it is not always the case that by reducing the expression of a central oscillator gene (or genes) abiotic stress tolerance will increase, for example, a *lhy/cca1* mutant shows decreased freezing tolerance (Dong *et al.,* 2011). These phenotypes are complex and are conferred by the clocks widespread influence on the transcriptome. Covington *et al.,* (2008) showed that 50% of genes that are DE in response to salinity, drought or heat and 40% of genes that are DE in response to cold display rhythmic expression patterns under free-running conditions.

1.4.3.1.1 Circadian gating of the abiotic stress response

The clock not only controls the transcriptional expression of these genes but also regulates the amplitude of the plants response to abiotic stresses by gating. For example, the *C-REPEAT BINDING FACTOR* (*CBF)* group of cold-responsive TFs regulates over 100 cold responsive genes (Thomashow, 1999). If a plant experiences cold stress on a morning (ZT 4), the *CBF*s driving cold responses are upregulated more compared to their basal levels than if that stress occurs later in the day (ZT 16) (Fowler *et al*., 2005) (as demonstrated in Fig.1.10). Moreover, 75% of all cold-responsive TFs were more highly induced in response to cold stress on a morning (A in Fig.1.10) compared to at night (B in Fig.1.10) (Bieniawska *et al.,* 2008).

Circadian gating of the transcriptional responses to abiotic stress (as illustrated in Fig.1.10) is a widespread phenomenon in Arabidopsis. This gating has been

observed in response to cold stress (as previously outlined), light stress (Takeuchi *et al.,* 2014) and drought stress (Wilkins *et al.,* 2010).

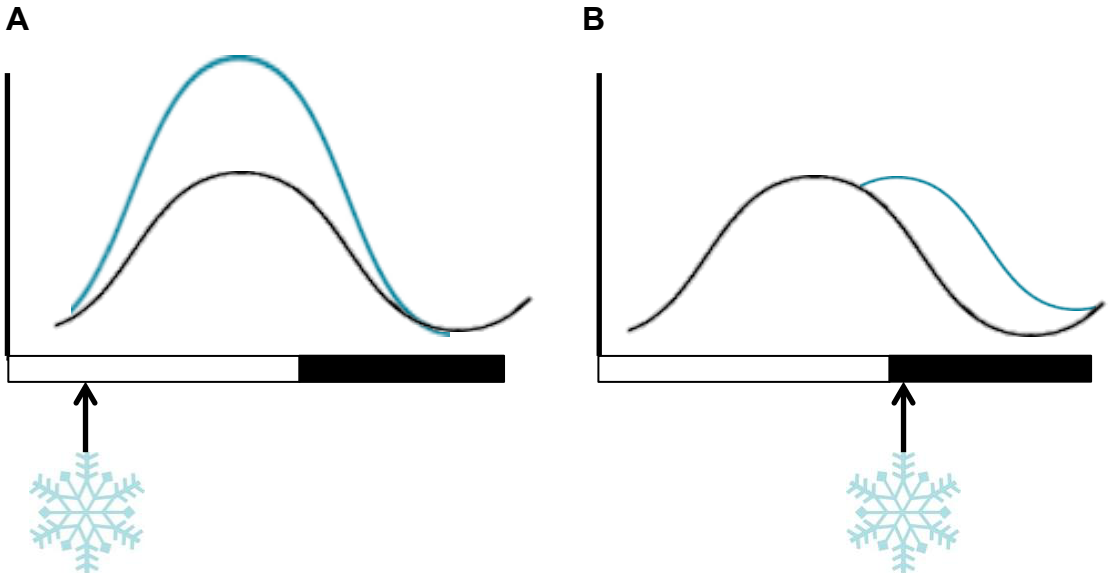


Figure 1.10 - Circadian gating of the abiotic stress response. *CBF*s are under circadian regulation and the clock also gates their response. A – Plant is stressed by cold at ZT 4 – CBF expression is then rapid and greatly induced. B – Plant is stressed by cold at ZT16 and CBF expression is increased but to a lesser extent due to circadian gating. Black line – basal expression levels. Blue line – induced expression levels in response to cold stress. White block – ZT0 – ZT 12 (day). Black box – ZT 12 ZT 24 (night). Snowflake– time cold stress applied. (Information for figure from Fowler *et al*., (2005))

1.4.3.1.2 Abiotic stress response, alternative splicing and the circadian clock

The core circadian clock genes have been shown to be differentially alternatively spliced in response to a range of abiotic stresses (Filkichkin *et al.* (2010); Filkichkin *et al.* (2012); Kwon *et al.* (2014); Seo et al. (2012)). *CCA1* is alternatively spliced to form one of two isoforms; CCA1α is the full-length protein whereas the CCA1β isoform is truncated with the MYB DNA-binding region is absent (Seo *et al.,* 2012). CCA1β suppresses the formation of CCA1α-CCA1α, CCA1α-LHY, and LHY-LHY functional heterodimers through competitive binding (Seo *et al.,* 2012). Inhibiting the formation of these heterodimers reduces the DNA binding capability of CCA1α and LHY and therefore dampens

circadian rhythms (Seo *et al.,* 2012). Interestingly, temperature also influences the levels of CCA1α and CCA1β isoforms synthesized, at low temperatures (4°C), 80% less CCA1β is formed compared to when temperatures are ambient (23°C) (Seo *et al.,* 2012). Furthermore, it was observed that plants constitutively expressing CCA1α or CCA1β had reduced or increased tolerance to freezing, respectively (Seo *et al.,* 2012). This tolerance was attributed to CCA1α positively regulating cold-responsive genes *GIGANTEA (GI*) and *CBF* (Seo *et al.,* 2012).

1.4.3.2 Role of the circadian clock in biotic plant defence

The plant circadian clock has a significant impact on the outcome of plant interactions with the biotic environment (Bhardwaj *et al.,* (2011); Goodspeed *et al.,* (2012); Goodspeed *et al.,* (2013); Wang *et al.,* (2011); Zhang *et al.,* (2013b)). However, how the plant clock modulates these interactions is poorly understood.

1.4.3.2.1 Herbivory

The feeding behavior of numerous insects is under circadian regulation (Chatterjee *et al.* (2010); Goodspeed *et al.* (2012)), with the feeding activity of *Trichoplusia ni* (cabbage loopers) increasing during the day and peaking at night (Goodspeed *et al.,* 2012). Plants have evolved to anticipate the time of day dependent increased appetite of the insects and regulate herbivory related defences accordingly.

Arabidopsis entrained under 12:12 LD conditions co-incubated with *T. ni* entrained to the same lighting phases (in-phase) show increased tissue loss at night when insect feeding activity peaks. This phenomenon persists under DD or LL conditions. This tissue loss is significantly enhanced if plants and insects are incubated in out of phase conditions (so when the plant perceives it to be subjective dawn the insect perceives it is subjective night) and then co-

incubated together (Goodspeed *et al.,* 2012). These susceptibility patterns suggest the plant can anticipate and account for the increased feeding behavior of the insect and adjust times of peak resistance accordingly. Given these resistance patterns persisted under free-running conditions (LL or DD) it can be assumed this is a circadian driven mechanism. However, to confirm this two circadian clock mutants (*CCA1-OX* and *lux2*) were subject to the same treatments as above and neither clock mutant showed any differences in levels of susceptibility to *T. ni* entrained in or out of phase with the plants (Goodspeed *et al.,* 2012).

Arabidopsis resistance to *T. ni* feeding requires a functional JA pathway (Kessler *et al.,* 2002). Consequently, it was hypothesized that the JA pathway could be contributing to the circadian mediated immunity and two mutants deficient in JA accumulation (*aos* and *jar1*) were subjected to the same experiment as above (Goodspeed *et al.,* 2012). Similar to the clock mutants, neither JA mutant exhibited any difference in tissue loss between insects entrained either in-phase or out of phase with the plants. This suggests that the clock regulates herbivory related defences at least in part through regulation of the JA pathway. This was confirmed when JA and SA levels were measured under 12:12 LD or LL conditions and JA peaked in the middle of the day when *T. ni* feeding activity was high (Goodspeed *et al.,* 2012).

The enhanced resistance of Arabidopsis entrained to the same light cycles as insects was termed 'circadian enhanced resistance' and has been shown to persist among many economically valuable crop species including lettuce, spinach, zucchini, sweet potatoes, carrots and blueberries (Goodspeed *et al.,* 2013). These crops were tested under post-harvest conditions suggesting that crop losses to herbivores could be decreased if LD entrainment was maintained during post-harvest transport and storage.

1.4.3.2.2 Biotrophic pathogens

Many *R*-genes involved in defence against the obligate biotroph, *Hpa*, have been shown to be under circadian regulation with diurnal expression patterns as well as evening element (EE) motif(s) in their promoter regions (Wang *et al.,* 2011). The presence of an EE motif is often an indicator that a gene is under direct circadian regulation (Harmer *et al.* (2000); Harmer and Kay (2005)). This observation formed the basis of the hypothesis that the clock could play a role in regulating the biotic defence response against biotic pathogens. To test this hypothesis, Wang *et al.* (2011) inoculated Arabidopsis at dawn or night and performed spore counts. Col-0 plants showed an enhanced number of spores after inoculation with *Hpa* at night compared to dawn (Wang *et al.,* 2011). Moreover, it was found that the circadian clock gene mutant (*cca1*) had no time of day dependent difference in susceptibility to *Hpa,* regardless of whether inoculations occurred at dawn or night.

Wang *et al*., (2011) observed a direct link between one component of the central oscillator, CCA1, and the defence response against *Hpa*. However, a link between the full central oscillator and the defence response was established by Bhardwaj *et al.,* (2011). The hemi-biotrophic pathogen, *P. syringae* strain DC3000, was used to inoculate Arabidopsis Col-0 plants at different times of day over a 48 h time period under CL conditions. Plants inoculated at subjective dawn (circadian time (CT) 26 or CT 50) showed a significantly reduced bacterial titre count 48 h after inoculation compared with those plants inoculated at subjective night (CT 42 or CT 46) (Bhardwaj *et al.,* 2011). Unlike the study by Wang *et al.,* (2011), this study was carried out under LL conditions, meaning all differences seen could be attributed to the clock and researchers could be certain this was not influenced by changes in response to light.

To confirm the time-of-day dependent differences observed were indeed clock driven, *CCA1-OX* mutants were inoculated at subjective dawn or subjective night and differences in bacterial titre observed. No differences were observed in bacterial counts between the two inoculation times confirming the link to CCA1.

To determine if this was an independent action of CCA1 or regulated by the central oscillator, a further clock mutant, *elf3-1* was employed. *elf3-1* was subjected to the same experiment at *CCA1-OX* and again displayed no time of day dependent differences in immunity (Bhardwaj *et al.,* 2011).

The clock was determined to be driving PTI responses to elicit the time of day dependent immunity. *P. syringae* hrpA (lacking a type III secretion system to inject effectors into the host cell) was used to inoculate plants on at subjective dawn or subjective night under LL and callose deposition was measured 14 hpi. Plants inoculated at dawn deposited significantly more callose than those inoculated at night showing the plant PTI response is more active in response to dawn inoculations (Bhardwaj *et al.,* 2011).

Further to the results obtained by (Bhardwaj *et al.,* 2011), a subsequent study observed a significant decrease in *P. syringae* pv. maculicola ES4326 strain DG3 bacterial counts between dawn or night inoculations after pressure infiltration under 12:12 LD or LL (Zhang *et al.,* 2013b). Pressure infiltrations inject bacteria directly into apoplast leaf space; however, under natural conditions bacteria exist on the leaf surface and enter via wounds or stomatal openings. When bacteria were spray-infected onto leaf surfaces at subjective dawn or night it was seen that a higher bacterial count was surprisingly obtained after Col-0 plants were inoculated at subjective dawn compared to a subjective night under 12:12 LD (Zhang *et al.,* 2013b). Concurrently plants sprayed with *P. syringae* had increased stomatal aperture at dawn compared to night (Zhang *et al.,* 2013b).

These results appear contradictory, however, the authors proposed that the clock is modulating the defence response on two levels; firstly by regulating stomatal aperture and secondly by mediating gene regulatory defences. Plants with increased stomatal aperture at dawn are more likely to be infected and those with smaller aperture at night are less likely. The clock then modulates a second layer of defence responses, which cause non-stomatal defences to be more active on at dawn to account for the open stomata and those defences at

night to be decreased as pathogens were unlikely to enter the cells due to smaller stomatal aperture. Hence, pressure infiltrated *P. syringae* bypasses the stomatal layer of defence and due to the decreased defences is more damaging to the host (Zhang *et al.,* 2013b).

Zhang *et al.,* (2013b) further explored the role of the clock in defence by screening circadian clock mutant plants for resistance to *P. syringae* and *Hpa*, with *lhy/cca1* double mutants displaying increased susceptibility to *Hpa* and *P. syringae* inoculation. *CCA1-OX* mutants showed increased resistance to *Hpa* inoculation and decreased resistance to *P. syringae* and *LHY-OX* lines showed decreased resistance to *P. syringae*. It was therefore concluded that the central oscillator coordinates the plant defence response against both hemi-biotrophic and obligate biotrophic pathogens. However, the exact mechanisms behind this regulation remain to be elucidated.

In agreement with Zhang *et al.,* (2013b), Panchal *et al.,* (2016) found that *P. syringae* infections with numerous strains resulted in a higher bacterial count after inoculation at dawn (under light conditions) compared to night (under dark conditions). Panchal *et al.,* (2016) then further investigated these results by investigating coronatine (COR) activity under different lighting conditions. COR is a JA-Ile mimic virulence factor produced by specific *P. syringae* strains to manipulate the host defence response (Melotto *et al.,* 2006). By mimicking JA-Ile, COR antagonizes the SA defence pathway which, as outlined, is the key hormonal pathway used by plants to defend against *P. syringae* (Melotto *et al.,* 2008). COR prevents PAMP induced stomatal closure and has been shown to reopen closed stomata (Melotto *et al.,* 2006).

Zhang *et al.,* (2013b) hypothesized that at night when stomata are closed plants reduce non-stomata related defences against pathogens, as pathogen attack is less likely. Panchal *et al.,* (2016) showed COR is produced approximately four h after *P. syringae* is inoculated on a leaf surface. This production was independent of light or dark conditions. However, COR did not induce stomatal opening under light conditions. Conversely, under dark conditions stomatal

opening was induced by COR applied independently or produced by a COR-producing *P. syringae* strain. This suggests that *P. syringae* COR interferes with circadian regulated stomatal closure at night. In doing so, the pathogen bypasses the plants main defence response to *P. syringae* at night, the closure of stomata.

1.4.3.2.3 Necrotrophic pathogens

Windram *et al.,* (2012) uncovered evidence that the clock may be contributing to the transcriptional defence response to *B. cinerea*. Uninfected Arabidopsis leaf tissue was harvested every 2 h over a 48-hour period; transcriptomic analysis of this tissue revealed 2404 genes exhibited a circadian expression profile. Of these, over 60% were differentially expressed in response to *B. cinerea* inoculation (Windram *et al.,* 2012).

This prompted simultaneous investigations by Hevia *et al.,* (2015) and Ingle *et al.,* (2015) (results presented in Chapter 3 and 4 of this thesis) into the influence of the circadian clock on the outcome of an interaction between *B. cinerea* and Arabidopsis. As outlined above, Hevia *et al.,* (2015) uncovered a circadian mechanism within *B. cinerea* B05.10. It was also found that the fungal clock could influence the outcome of the *B. cinerea* and Arabidopsis interaction.

In agreement with results obtained by Hevia *et al*., (2015), Ingle *et al.,* (2015) found Col-0 plants entrained under 16:8 LD dark conditions for 4 weeks then inoculated with *B. cinerea* strain pepper under LD or LL conditions also exhibited maximal resistance in after dawn inoculations. The *B. cinerea* employed by in this thesis had been incubated in constant darkness and constant temperature so received no circadian entrainment and was a different strain to that employed by Hevia *et al.,* (2015).

Ingle *et al.,* (2015) inoculated two circadian clock mutants (*cca1 lhy* and *elf3-1*) at subjective dawn or subjective night under LL conditions after 4 weeks of LD entrainment. It was observed that both mutants lost any differences between

lesion sizes from inoculations occurring at the two different times of day (dawn and night). Again the fungus employed received no entrainment showing the differences previously observed to be driven, at least in part, by the circadian clock in plants.

Ingle *et al*., (2015) and Hevia *et al,* (2015) have shown a significant difference in susceptibility to the necrotrophic, fungal pathogen, *B. cinerea*, when inoculated at subjective dawn compared to subjective night. The circadian clock governs these differences as they persistent under free-running conditions and are abolished in dysfunctional clock mutants. From here, the pivotal question was how is the clock modulating these defences? Given the role the circadian clock has in regulating plant hormones as well as the prominent role plant hormones play in both abiotic and biotic defences, links between the clock and phytohormones will be investigated.

1.4.3.3 The circadian clock and phytohormone regulation

The circadian clock plays a role in the biosynthesis/signalling/metabolism of some, if not all phytohormones. However, discussion here will be limited to SA, JA and ET, since these are arguably most relevant to plant-pathogen interactions.

SA synthesis is regulated by the circadian clock and alongside JA is thought to have a role in the time of day dependent differences in biotic responses against herbivory and biotrophic pathogens (Goodspeed *et al.* (2012); Goodspeed *et al.* (2013); Zheng *et al.* (2015)). The mechanism by which the clock regulates SA levels is unknown and very few links have been uncovered to date. One way in which the clock could be influencing SA production is through a Phosphate Transporter (*PHT4;1*) that negatively regulates SA accumulation and is under circadian-clock regulation (Wang *et al.,* 2014). However, the precise mechanism by which *PHT4;1* could be influencing SA production remains unknown at this stage.

Another way the clock could be regulating SA synthesis is through *CCA1 HIKING EXPEDITION (CHE)* (also known as *TCP21*) binding upstream of the TSS of a gene encoding a key SA biosynthetic enzyme, *isochorismate synthase 1 (ICS1)* (Zheng *et al.,* 2015). SA levels are known to fluctuate over a 24h period and under DD conditions, SA levels peak in the middle of the subjective night (Fig.1.11) (Goodspeed *et al.,* 2012). *CHE* positively regulates SA biosynthesis and a *che-2* mutant displayed reduced and constitutive production of SA, demonstrating that this interaction is key to maintaining the rhythmicity of SA biosynthesis (Zheng *et al.,* 2015).

ET biosynthesis is also under circadian regulation in Arabidopsis (Thain *et al.,* 2004). This regulation most likely occurs through the regulation of a key ET biosynthetic enzyme, *ACC SYNTHASE 8 (ACS8)* which when mutated causes ET production to lose its rhythmicity (Thain *et al.,* 2004). *ACS6* and *ACS8* were found to display circadian expression patters and TOC1 has been shown to bind to sequences upstream of *ACS6* and *ACS8* TSS (Covington *et al.,* (2008); Grundy and Stoker *et al.,* (2015)). Moreover, *EIN3* has a prominent role in ET pathogen-related host defences (Alonso *et al.,* 2003) and has previously been shown to be under the control of *XAP5 CIRCADIAN TIMEKEEPER (XCT)* (Ellison *et al,* 2011).

JA biosynthesis is tightly regulated by the circadian clock in Arabidopsis and under non-stress conditions JA levels peak in the middle of the day in contrast to SA which peaks in the middle of the night (under DD conditions after 12:12 LD entrainment) (Fig.1.11) (Goodspeed *et al.,* 2012).

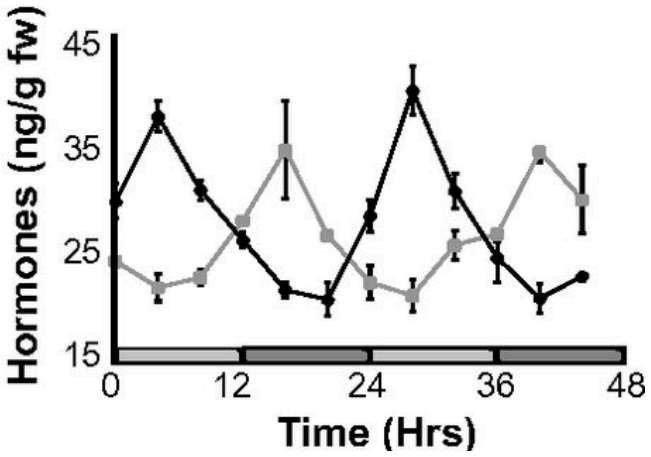


Figure 1.11 – Jasmonate and salicylate accumulation patterns are circadian-regulated with opposite phasing. Jasmonates peak in the middle of subjective day (black line) and salicylates (gray line) peak in the middle of subjective night. Mean ± SE; *n* = 3. fw, fresh weight. JA/SA levels. Constant DD 12:12. Black – JA. Grey – SA (Reproduced with permission from Goodspeed et al., 2012)

It has previously been shown that JA responsive genes (genes significantly up or downregulated following MeJA treatment) are significantly overrepresented for rhythmic expression patterns (Mizuno *et al.,* 2008). Several rhythmically expressed genes upregulated in response to MeJA treatment peaked in expression at ZT 12 under 12:12 LD conditions (Mizuno *et al.,* 2008). This peak in expression agrees with the finding by Windram *et al.,* (2012) that under 16:8 LD conditions JA signalling genes peak in expression between ZT 10-12 in Arabidopsis leaves. As can be seen in Fig.1.11, JA levels peak just prior to ZT 10, at approximately ZT 6.

In addition to the circadian-regulated changes in both JA hormone levels and gene expression of JA-responsive genes, the circadian clock has also been shown to gate the JA signalling pathway, in a similar manner to that shown in Fig.1.10 (Shin *et al.,* 2012).

MYC2 is a key regulator of JA defences (Kazan and Manners, 2013). MYC2 protein and transcript levels display circadian rhythmicity under 12:12 LD conditions, with the transcript levels peaking at ZT 12 and protein abundance peaking between ZT 4 – ZT 12 (Shin *et al.,* 2012). *TIME FOR COFFEE* (*TIC*) was shown to directly bind to and inhibit MYC2 protein accumulation (Shin *et al.,*

2012). Shin *et al*., (2012) also observed that MYC2 protein accumulation increased in response to MeJA application if the MeJA treatment occurred at ZT 0, but if the treatment occurred at ZT 12, a reduced increase was observed. This is an example of circadian gating of responses. TIC is directly regulating the JA signalling pathway by gating the response of MYC2 protein accumulation in response to JA levels. It is possible that additional clock genes are also interacting with other genes involved in the MYC2 pathway (such as *JAZ1, JAZ5, JAZ6* and *JAZ9* (Appendix 1)) to contribute to this gating response.

Why the clock would be gating this specific JA pathway in such a regulated and complex manner is yet to be elucidated. The circadian clock and phytohormones both play major roles in fine-tuning the internal biology of an organism to the external environment so cross talk between them is vital. It is likely that the JA signalling stress response is highly costly in terms of energy expenditure and therefore having high levels at a particular time of day without any stress stimuli is wasteful.

1.9 Aims

The overall aim of this project is to elucidate how the plant circadian clock modulates defence responses against *B. cinerea*.

1. To investigate differences in transcriptional changes induced by *B. cinerea* inoculation at two key times of day, when plants are exhibiting different levels of resistance to *B. cinerea*.

2. To investigate the mechanisms by which the circadian clock is impacting upon the defence response by:

- Identifying proteins acting as links between the plant circadian clock and the defence response.
- Optimize a plant based ATAC-Seq protocol to allow TF occupancy to be investigated at the different times of day before and after *B. cinerea* inoculation.

Chapter 2: Materials and Methods

2.1 Plant and fungal material

2.1.1 Plant growth

Arabidopsis thaliana seeds were stratified in 0.1% agarose at 4°C for 72 hours (h) in darkness. Stratified seeds were sown in pre-soaked Arabidopsis compost mix (6:1:1 Levington F2 compost:sand:vermiculite) in 4 cm^2 pots (P24, Desch Plant-pak). Pots were covered in cling film to create a humid environment and placed in a growth chamber to germinate. Seven days after sowing the covering was removed and seedlings were thinned to one seedling per pot. Plants were grown in standardised conditions of 16-h days, 22°C temperature, 68% relative humidity, 350 ppm CO^2 and 100 $\mu mol.m^2.s^{-1}$ light.

2.1.1.1 Plant growth conditions for *Botrytis cinerea* inoculations

Plants to be inoculated at subjective dawn (Zeitgeber (ZT) 0)) or subjective night (ZT 18) with *B. cinerea* under free-running conditions were grown for four-weeks in two Sanyo 970 cabinets with light settings 'out of phase' so they could be inoculated with the same fungal inoculum (Table 2.1). Inoculations took place at ZT 0 or ZT 18 and the light cycling continued. Plants to be inoculated at subjective dawn (circadian time (CT) 24)) or subjective night (CT 42) with *B. cinerea* under constant light (LL) conditions were grown for four-weeks under the conditions outlined in Table 2.1, 24 h prior to inoculation the plants in both cabinets were placed under constant light conditions, inoculated at CT 24 or CT 42 and kept under LL for the remainder of the inoculation. Both cabinets had the same humidity, light levels, temperature and CO^2 levels as outlined above.

Table 2.1 – Out of phase cabinet settings used for the subjective morning and subjective night inoculations. ZT – Zeitgeber time (time as perceived by the plant). ZT0 – lights come on. ZT16 – lights go off. Green – time of *B. cinerea* inoculation.

ZT	Cabinet one	ZT	Cabinet two
0/24	on	18	off
1	on	19	off
2	on	20	off
3	on	21	off
4	on	22	off
5	on	23	off
6	on	0/24	on
7	on	1	on
8	on	2	on
9	on	3	on
10	on	4	on
11	on	5	on
12	on	6	on
13	on	7	on
14	on	8	on
15	on	9	on
16	off	10	on
17	off	11	on
18	off	12	on
19	off	13	on
20	off	14	on
21	off	15	on
22	off	16	off
23	off	17	off

2.1.2 Transgenic plant material

Arabidopsis Columbia 0 (Col-0) lines were ordered from the European Nottingham Arabidopsis Stock Centre (NASC) or from the original authors (Table 2.2).

Table 2.2 – Details of transgenic lines. Details of all transgenic lines employed during this research. Details include how the mutant is referred to in the text, full identification details and source of seeds. Triple mutants were generated as described in de Torres Zabala *et al.* (2015). All lines are in a Col-0 background. N/A - not applicable.

Mutated gene(s)	Plant mutant name in text	ID or T-DNA insert	Source
ein3	*ein3-1*	ID: CS8052 (Chao *et al.*, 1997)	NASC
jaz6	*jaz6*	SAIL_1156_C06	NASC
jaz5	*jaz5*	SALK_053775	NASC
jaz10	*jaz10*	SAIL_92_D08	NASC
jaz7	*jaz7*	WiscDsLox7H11	NASC
Jaz6 jaz7	*jaz6.7*	N/A	Prof. Murray Grant de Torres Zabala *et al.* (2015)
Jaz7 jaz10	*jaz7.10*	N/A	Prof. Murray Grant de Torres Zabala *et al.* (2015)
Jaz5 jaz10	*jaz5.10*	N/A	Prof. Murray Grant de Torres Zabala *et al.* (2015)
jaz5 jaz6 jaz10	*jaz5.6.10*	N/A	Prof. Murray Grant de Torres Zabala *et al.* (2015)
jaz5 jaz7 jaz10	*jaz5.7.10*	N/A	Prof. Murray Grant de Torres Zabala *et al.* (2015)
jaz6	*JAZ6-OX*	*35S:JAZ6:GFP*	Prof. Ari Sadanandom Unpublished. Generated at Durham University. Vector used: pEarlygate 103. Selection: BASTA.

2.1.3 *B. cinerea* culturing and harvesting

B. cinerea strain pepper (Denby *et al.,* 2004) or strain B05.10 were cultured on sterile tinned apricot halves (Del Monte) in petri dishes at 25°C in constant darkness, with sub culturing every two weeks. *B. cinerea* spores were harvested after 14-21 days of growth by scraping spores from three apricot halves into 3 mL of sterile water in a class II flow cabinet. Spores were filtered through glass wool to remove mycelium and the concentration diluted to 2 x 10^5 spores/mL before being diluted by a 1:1 ratio of sterile grape juice to a final concentration of 1 x 10^5 for use.

2.1.4 Phenotyping of Arabidopsis plants to *B. cinerea* infection

Three leaves (leaves: 7, 8 and 9) from four-week-old Arabidopsis plants were detached and placed on 0.8% w/v phyto-agar propagator trays (approximately 100 leaves per tray) containing a 5 cm reference measure. At least 30 leaves were used per plant line (3 leaves per plant), and each tray held 10 leaves per plant line. A 5 µL droplet of 1 x 10^5 *B. cinerea* inoculum was placed in the centre of the surface of each leaf. Propagator trays were covered with lids, sealed with tape, placed in a Sanyo 970 and kept under the same conditions as for plant growth, except the humidity was increased to 80%.

To phenotype plants photographs were taken of propagator trays at 24-hour increments post inoculation (24 hours post inoculation (hpi), 48 hpi, 72 hpi). A 5 cm scale measure was included in each propagator tray. Lesion sizes were calculated using the scale measure in ImageJ (Abràmoff *et al.,* 2004). Student's T-Test was then used to determine significant differences between lines and also between leaves inoculated at different times of day (e.g. ZT 0 compared to ZT 18).

2.1.5 *B. cinerea* inoculations for gene expression or phytohormone analysis

Fungal inoculums for gene expression assays were prepared as outlined above. Plants had leaf seven identified on emergence by placing a toothpick close to the leaf. Leaf 7 was then detached and placed on a propagator tray as previously outlined. Five 5 µL drops of *B. cinerea* inoculum were dropped on to each leaf surface with even spacing between droplets. Mock treatments were performed in an identical manor except the inoculum used was grape juice diluted with sterile water at a 1:1 ratio. Mock and infected leaves were then incubated as outlined above for phenotyping assays. Samples were then harvested at the appropriate time points by flash freezing and then stored at -80°C. Exactly the same process was carried out on leaves 5 and 6 when tissue was intended for phytohormone extraction.

2.1.6 Trypan blue staining of *B. cinerea* inoculated leaves

Inoculations were carried out as outlined for gene expression assays. Whole leaves were then harvested at the specified time points by placing samples directly into 1 mL of lactophenol-trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol, 0.02 g trypan blue, 10 mL distilled water) and then boiled for 1 minute at 90°C, then left overnight in the stain. Destaining was carried out by placing leaves in 1 mL of chloral hydrate (2.5 g of chloral hydrate in 1 mL of distilled water) overnight (similar to what is outlined in: Koch and Slusarenko 1990). Destained leaves were mounted in water and 1% tween and viewed using a light microscope.

2.2 Molecular methods

2.2.1 Genotyping plant mutants

Table 2.3 - Primers used to genotype JAZ mutant lines.

Gene	T-DNA insert	Primer sequence
JAZ5 (AT1G17380)	SALK_053775	F- ACGTTCCACGATCTGATTTTG R- GTACTCTTCCATTTTACGCGC
JAZ6 (AT1G72450)	SAIL_1156_C06	F- TTTGCAAATGCCCTCATTTAC R- TTAGAACAGAAATTGCAAACCG
JAZ7 (AT2G34600)	WiscDsLox7H11	F- GGACGAATCAAGCAGCTCTG R- GGGGAAGTTGCTTGAATCCG
JAZ10 (AT5G13220)	SAIL_92_D08	F- CCGTCTTTTCTGGTTTGGCA R- ACTCAAAGTCAACGCGCTTT

All knockout lines in Table 2.2 were genotyped using gene specific primers (Table 2.3) along with the appropriate T-DNA specific primer (SALK LBb1.3 ATTTTGCCGATTTCGGAAC, SAIL LB1-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC or WiscDsLox-AACGTCCGCAATGTGTTATTAAGTTGTC). *JAZ6-OX* was genotyped using a forward primer designed around the *35S* promoter –CTATCCTTCGCAAGACCCTTC and the reverse primer designed specifically to the *JAZ6* gene –AAGCTTGAGTTCAAGGTTTTTGG. DNA extractions and subsequent

PCR amplifications were performed using the RedExtract-N-Amp Kit (Sigma-Aldrich) following manufacturer's instructions.

2.2.2 Gel electrophoresis

Visualisation of DNA separation was achieved by running samples on a 1% electrophoresis gel at 120 V for approximately 40 minutes. Agarose gels were typically comprised of 1% ultrapure agarose (Invitrogen), 1 x TAE buffer (40mM Tris base, 20mM acetic acid, 1mM EDTA, pH8.0) and 1 x GelRed (Biotium). Hyperladder I (Bioline) was used as a DNA size marker in all gels.

2.2.3 RNA extraction

After storage at -80°C samples then had 2 x 6mm glass beads added and leaves were homogenised using a Mixer Mill MM 400 (Retsch) at 25 W for 1 minute, if tissue was not fully homogenised this grinding process was repeated. Samples were then flash frozen and 1 mL Trizol (Invitrogen) added, samples quickly vortexed then incubated room temperature (RT) for 5 minutes. Following this, 200 µL of chloroform (Fisher) was added, samples quickly vortexed and incubated at RT for 3 minutes then centrifuged at 14,000 × g for 15 mins at 4°C. The supernatant was then transferred to a new Eppendorf and 70% ethanol (70% ethanol (Fisher) in DEPC treated water) added and samples well mixed. Each sample then each added to an RNeasy purification column (QIAgen) and purified following the manufacturer's instructions. 35 µL DEPC treated water was added to column to elute the sample; the concentration of the sample was then quantified by adding 1.5 µL to a Nanodrop ND-1000 spectrophotometer (Thermo scientific).

2.2.4 cDNA synthesis

Removal of genomic DNA from extracted RNA was performed as follows, 1 µg of RNA was treated with 1 µL of DNAse I (Sigma-Aldrich) and 1 µL of 10 x DNAse I buffer (Sigma-Aldrich) and the final volume made up to 10 µL using DEPC treated water. This solution was incubated for 15 mins at RT prior to the addition of 1 µL of stop solution (Sigma-Aldrich) and a 10 minute 70°C incubation.

cDNA synthesis was then carried out using Moloney Murine Leukemia Virus (M-MLV) Reverse transcriptase (Promega) following the manufacturer's instructions. In brief; 1.25 µL 10mM dNTPs (Promega), 5 µL M-MLV 5 x Reaction Buffer (Promega), 25 units of Recombinant RNasin Ribonuclease Inhibitor (Promega), 200 units of M-MLV Reverse transcriptase were added to the DNase treated RNA sample and the final volume made up to 25 µL using distilled water. Each sample was gently mixed and incubated at RT for 10 mins, 42°C for 15 mins, followed by a final incubation of 37°C for 60 mins. cDNA was then stored at -20°C.

2.2.5 qPCR

Table 2.4 - Primers used in qPCR to detect *JAZ6* and *PUX1* transcript expression levels. Annealing temperatures used and amplicon sizes are outlined.

Gene	Primer sequence	Annealing temp (°C)	Amplicon size (bp)
JAZ6 (AT1G72450)	F- ACAGGGCTGTGGCTAGAG R- CTTTCTTGTCCACCTCCATC	55	171
PUX1 (AT3G27310)	F- TTTTTACCGCCTTTTGGCTA R- ATGTTGCCTCCAATGTGTGA	60	149

Primers and annealing temperatures used to quantify the expression levels of *JAZ6* in Col-0 and mutant plants can be found in Table 2.4 alongside the primers used to measure the *PUX1* transcript levels (AT3G27310). The mRNA levels of *PUX1* are unaffected by *B. cinerea* infection so this was used as a reference gene for each sample (Windram *et al.* 2012).

5 ng of cDNA was mixed with 5 µL SsoAdvanced SYBR Green Supermix (Bio-Rad) and forward and reverse gene specific primers (3 mM), the final volume was then made up to 10 µL using distilled water. Each primer mix has a non-template control containing no cDNA template to ensure no contamination was influencing results. Each reaction was carried out in triplicate to account for technical variation. A standard curve was employed for each primer set by

mixing equal volumes of each sample in the reaction, before 4 serial dilutions (1:10).

All qPCR was carried out using a 96-well Stratagene Mx3005P (Agilent) in a neutral 96-microwell plate (Fisher-Scientific) with ultra-clear, capped, polypropylene lids (Fisher-Scientific). Prior to entering the temperature cycles, samples were incubated at 95°C for 3 mins, samples then underwent 40 cycles of 95°C for 10 secs (denaturation) then 60°C for 30 secs (annealing/extension). Instrument default settings were then employed to carry out a post-cycle melt curve analysis, which was visually analysed.

2.2.5.1 qPCR analysis

qPCR data was analysed using the double delta Ct method as outlined in Livak *et al.,* (2001).

2.3 Measuring phytohormone levels

Tissue was flash frozen then ground using Mixer Mill MM 400 (Retsch) at 25 W for 1 minute; as before, if tissue was not homogenised this grinding process was repeated. SA, ABA and JA were extracted from 10 mg of homogenized frozen tissue as described in (Forcat *et al.,* 2008). Hormone measurements were performed using UPLC (Ultra-high Pressure Liquid Chromatography)-electrospray ionisation/MS-MS (Xevo TQ-S with NanoAcquity (both: Waters)) (carried out by Dr. Susan Slade, Genomics Facility, School of Life Sciences, University of Warwick). For each sample 3 technical replicates were performed with a solvent blank (10% methanol 1% acetic acid) ran through the UPLC-MS/MS between triplicates to clear the column. An internal standard (IS) of 10 ng of $^{2}H_{2}$ JA was added to each sample.

2.3.1 Analysing phytohormone level measurements

Levels of JA, SA and ABA were quantified using the TargetLynx program on MassLynx (Waters). This program calculates the area of the peak given off from

samples relative to the IS and then uses this to calculate the exact hormone levels per 10 mg of plant tissue. The equation employed by TargetLynx to calculate this is as follows:

(peak area of target/IS area) x standard concentration of IS on column = pg on column

Peak area of target is directly inferred from the chromatogram obtained from the MS-MS reading. *IS area* is again derived directly from the MS-MS chromatogram reading and references to a known quantity of the IS added during the hormone extraction process (Forcat *et al.,* 2008). *Standard concentration on column* refers to the concentration of internal standard known to be passing through the column.

To ascertain the total pg in a 10 mg sample, the pg on column figure is multiplied by the fraction of sample injected (*pg on column x fraction of sample injected* (in this case 1/80th was injected so this figure was 80)).

2.4 Yeast 2 Hybrid

The Yeast 2 Hybrid (Y2H) system detects protein-protein interactions. Methods employed are similar to that outlined in Dreze *et al.,* (2010). Each protein is individually cloned into either a 'prey' or a 'bait' vector (Table 2.5). The 'prey' vector (pDEST22) contains a *TRP1* gene that allows transformed yeast to grow on media lacking tryptophan. The 'bait' vector (pDEST-DB) contains a *LEU2* gene allowing transformed yeast to grow on media lacking in leucine. Moreover, an extra reporter gene system exists in each vector; each one contains different 'sections' of the *GAL4* expression system (Fig.2.1): pDEST22 contains the *GAL4* activation domain (AD) fused to the TF (blue in Fig.2.1), whereas pDEST-DB contains the *GAL4* binding domain (BD) fused onto the JAZ protein (pink in Fig.2.1). Once the two haploid yeast strains mate to form a diploid cell, if the JAZs and TFs directly interact (as illustrated by the black arrow in Fig.2.1) then the *GAL4* AD will 'switch on' the expression of the reporter gene, in this

research these are *HIS3* and *ADE2* (Fig.2.1). *HIS3* allows the diploid cell to grow on media lacking in histidine and *ADE2* allows the diploid cell to grow on media lacking adenine.

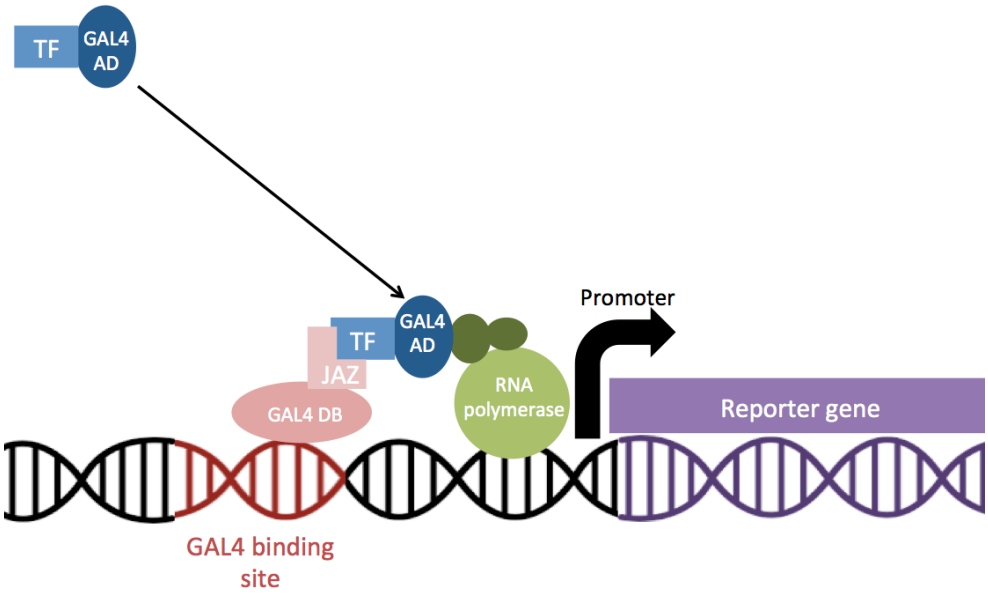


Figure 2.1 – Y2H reporter gene expression system. Both the 'bait' (pDEST-DB) and the 'prey' (pDEST22) vectors contain individual sections of the *GAL4* expression system. The bait vector contains the *GAL4* AD fused to the TF N terminus (AD: TF) (blue). The prey vector contains the *GAL4* DB fused to one of the JAZ proteins N terminus (DB: JAZ) (pink). If the TF and JAZ bind the GAL4 expression system will be 'turned on' and activate the RNA polymerase (green) to transcribe the reporter gene (purple) (*HIS3* or *ADE2*)

2.4.1 Sequence verification

Sequences within plasmids were verified by preparing 300-500 ng of plasmid DNA in 5 µL and combined with 5 µL of primers (M13 forward – GTAAAACGACGGCCAGTC or M13 reverse – AACAGCTATGACCATG). Samples were sequenced at GATC Biotech centre for sequencing.

2.4.2 Yeast 2 hybrid library information

2.4.2.1 JAZ protein library

The open reading frame (ORF) of JAZ genes (JAZs: 1, 2, 3, 4, 5, 6, 7, 8, 10, 11) was individually cloned into pDEST-DB (bait) vectors (Table 2.5) in the yeast

strain Y8930 (Dreze *et al.,* 2010) (provided by Dr. Sarah Harvey, School of Life Sciences, University of Warwick).

2.4.2.2 Transcription factor library

Pruneda-Paz *et al.,* (2014) created an extensive clone-collection of Arabidopsis transcription factors (TFs) in a yeast 1 hybrid (Y1H) compatible plasmid (pDEST22) in the yeast strain (YM4271). This library was used to transform an Y2H compatible yeast strain (AH109) using the same pDEST22 vector (total: 1956) (carried out by Dr. Ana Dominguez-Ferreras, School of Life Sciences, University of Warwick).

Table 2.5 – Vectors used for protein expression in *Saccharomyces cerevisiae.* Information on the uses, selective agents and references for each vector are included.

Protein domain	Vector name	Information	Selection in yeast	Reference
Binding domain (Bait)	pDEST™-DB	Generation of the binding domain plasmid in Y2H. GAL4 DB.	Leucine	Dreze *et al.,* (2010)
Activation domain (Prey)	pDEST™22	Generation of the activation domain plasmid in Y2H. GAL4 binding site: *GAL4* activation sequence.	Tryptoph-an	Life Technologies ™, Carlsbad, California, USA

2.4.3 Mating of the binding domain and activation domain strains

The bait strains were mated with the TF library prey strains on YPDA media, as described in (Dreze *et al.,* 2010). Diploid cells were formed after 24 h incubation at 30°C.

2.4.4 Replica plating on to selective media

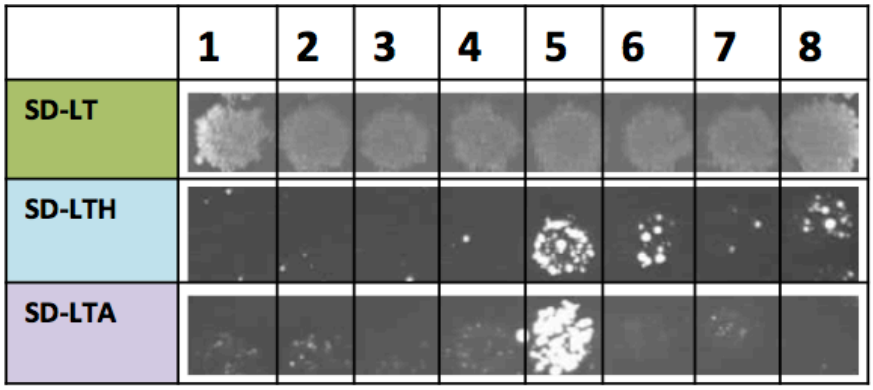
The YPDA mating plates were then replica plated onto selective medias (SD),

SD-Leu-Trp (SD-LT), SD-Leu-Trp-His (SD- LTH) and SD-Leu-Trp-Ade (SD-LTA) and incubated at 30°C for 24 h. SD plates were then replica cleaned and incubated at 30°C for 72 h, similar to what is outlined in Dreze *et al*., (2010) and Hickman *et al.,* (2013). 3-Amino-1, 2, 4-triazole (3AT) (a competitive inhibitor of the *HIS3* reporter gene) was not employed in this screen as two reporter genes were used, Dr. Ana Dominguez-Ferreras and Pruneda-Paz *et al.,* (2014) had not previously recorded any auto activation (self-activation of the *HIS3* gene from the activation domain strain) in the TF library and no auto-activation of the reporter genes was observed.

2.4.5 Identifying protein to protein interactions

Colonies can only grow on SD-LT if both strains have successfully mated and created a diploid cell with both *LEU2* and *TRP1* genes, giving colonies the ability to grow on media lacking in leucine and tryptophan, respectively. After successful mating was established, selective plates were checked for growth on SD-LTH and SD-LTA plates, growth on these is only possible if the protein in the prey vector binds with the protein in the bait vector activating the *HIS3* or *ADE2* reporter genes (Fig.2.1).

All plates were photographed using G:Box EF2 (Syngene, Cambridge, UK) on the upper white light setting and scored for growth. The scoring system employed can be seen in Fig.2.2. Unless otherwise stated, each interaction was repeated 3 times with independent YPDA mating reactions.



Scores	1	2	3	4	5	6	7	8
SD-LTH	0	0	0	0	0	0	0	0
SD-LTA	0	0	0	0	0	0	0	0

Figure 2.2 - Scoring of Y2H protein-protein interactions. Interactions were scored based on colony formation on media lacking in histidine (SD-LTH) and adenine (SD-LTA). Individual colonies were scored out of the 3 (0= no growth, 1= weak, 2= moderate, 3= strong). Any colonies with low growth on the SD-LT plate were repeated.

2.5 ATAC-Seq

Assay for Transposase-Accessible Chromatin coupled with high throughput sequencing (ATAC-Seq) was employed to map chromatin accessibility throughout the genome. Three replicate samples were harvested at both ZT 4 and ZT 10. For each sample 2 g of fresh 4-week-old Col-0 leaf tissue was harvested.

2.5.1 Nuclear enrichment

Nuclei extraction was performed using CelLytic PN Isolation/Extraction kit (Sigma) as outlined in manufacturers instructions. In brief, 6 mL NIB Buffer was ground with 2 g sample using a pestle and mortar. The sample was pelleted and washed several times (in NIB Buffer + 1% Protease Inhibitor Cocktail (Sigma-Aldrich) + 0.3% Triton X-100 (Sigma-Aldrich)) until chloroplasts removed.

Viable nuclei were then counted using DAPI (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich) (as outlined in Chazotte, 2010).

2.5.2 Transposase reaction

The transposase reaction was performed using Nextera DNA library preparation kit (Illumina). Nuclei were diluted to 30,000 nuclei per 25 µL Nextera Tagment DNA Buffer. 20 µL of nuclease free water and 5 µL of Transposase enzyme was added to the diluted nuclei and it was then incubated for 60 mins at 37°C.

2.5.3 DNA clean-up

After the transposase reaction potential PCR inhibitors remained, so DNA was cleaned using a DNA clean & concentrator kit (Zymo Research), following what is outlined in manufacturer's instructions (with the following specifications; DNA Binding Buffer used at a 1:3 ratio with the sample, followed by 3 wash steps using 200 µL of wash buffer and elution with 23 µL of elution buffer).

2.5.4 Indexing, PCR amplification and clean up

Sample indexing performed using Nextera DNA Sample Preparation Kit (Illumina) with the following PCR reaction volumes; 5 µL forward and reverse Nextera Indexing Primers (Table 2.6), 15 µL NPM, 5ul PPC and 20 µL eluted DNA sample. PCR conditions are outlined in Table 2.7.

Table 2.6 – Nextera primers employed for ATAC-Seq sample indexing. Primers had to be used in a specific combination- always a 500 primer in combination with a 700 primer, for more information see the Illumina Nextera primer guide.

Nextera Primer	Sequence
503	TATCCTCT
504	AGAGTAGA
701	TAAGGCGA
702	CGTACTAG
705	GGACTCCT
706	TAGGCATG

Table 2.7 – PCR Conditions employed for ATAC-Seq sample indexing and amplification. Similar conditions to those outlined in the Illumina Nextera primer guide.

Time	Temperature (°C)	Cycles
3 minutes	72	1
30 seconds	98	1
10 seconds	98	15
30 seconds	63	
3 minutes	72	

Following the PCR amplification, the samples were cleaned up using AMPure XP Beads (Beckman Coulter) following the manufacturer's protocol. 30 µL of AMPure XP Beads were added per 50 µL sample, this was then incubated at RT for 5 mins then placed onto magnetic rack for 2 mins. The supernatant was discarded and beads washed twice with 80% ethanol. Followed by a 15 min air dry. Samples were then re-suspended in 33 µL of 1 x TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA).

2.5.5 DNA quantification

Following on from the PCR clean up, DNA was quantified using a Quibit machine and fragment length distribution analysed using a Bioanalyzer (Agilent) (performed by Lesley Ward, Genomics Facility, School of Life Sciences, University of Warwick).

2.5.6 Sequencing

Four samples were diluted and pooled to 10 nM; quality checked (Wellcome Trust Sequencing Centre) and ran on one lane of the Illumina HiSeq 4000 with a 75 bp-paired end run at the Wellcome Trust Sequencing Centre.

2.6 *In-silico* methods

2.6.1 Nimblegen microarray data analysis

Experimental set up, production of the microarray data and normalisation techniques were performed by members of Katherine Denby's (School of Life Sciences, University of Warwick) or Robert Ingle's group (Department of Molecular and Cell Biology, University of Cape Town). Detailed in Ingle *et al.,* (2015).

2.6.1.1 Identifying significantly differentially expressed genes

All microarray analysis methods for gene expression changes were carried out by myself and details can be found in Ingle *et al.,* (2015). In brief, a 3-factor ANOVA was performed to identify genes differentially expressed in response to time of inoculation and inoculation with *B. cinerea* and Bonferroni multiple testing correction applied. This non-redundant gene list was then filtered for genes showing a $\geq \log_2 0.6$ change in expression in response to *B. cinerea* inoculation at either 18 hpi or 22 hpi. Further filters were then applied to identify genes in this list not only changing in response to inoculation but also changing in response to time of day when inoculation occurred. Genes showing a $\geq \log_2 0.6$ change between infected samples inoculated on a subjective morning compared to a subjective night and/or a $\geq \log_2 0.6$ difference in the ratio of infected: mock when inoculated at on a subjective morning compared to a subjective night. A final list of 901 genes was selected (Ingle *et al.,* (2015) - Supplementary data set 2).

2.6.1.2 Grouping and analysing significantly differentially expressed genes

In order to gain biological insight into the 901 gene list, it was grouped by expression changes, again this grouping is detailed in Ingle *et al.,* (2015). These eight groups are further detailed within Chapter 3.

2.6.1.2.1 GO Term enrichment

These eight groups were then subjected to overrepresented Gene Ontology (GO) Term analysis using BiNGO 2.44 (Maere *et al,* 2005) hypergeometric test with a Benjamini and Hochberg False Discovery Rate (p-value <0.05) applied for overrepresented GO Biological terms in. Updated TAIR annotations for Arabidopsis were downloaded from geneontology.org.

2.6.1.2.2 Motif enrichment

The same eight groups were then tested for overrepresentation of TF binding motifs in the promoter regions (corresponding to 500bp upstream of the transcription start site) of genes; this was carried out using MEME-LaB (Brown *et al.,* 2013). MEME-LaB identifies known motifs by providing a detailed comparison to those stored in the following databases; JASPAR (Bryne *et al.*, 2008), PLACE (Higo *et al.*, 1999) and TRANSFAC (Matys *et al.,* 2006). For each group the top 5 overrepresented motifs were retrieved using this tool, overrepresentation ranking explained in Brown *et al.,* (2013).

Motif overrepresentation was also tested for using more recently published information on TF binding motifs (Weirauch *et al.,* (2014) and Franco-Zorilla *et al.,* (2014)). This was carried out using a similar method to what is outlined in Breeze *et al.,* (2011) with the addition that in preparation for the binomial test, the individual motif *p*-values were combined via the Hommel method (Hommel, 1983) (program designed by Dr. Krzysztof Polanski, Warwick Systems Biology Centre, University of Warwick).

Chapter 3: Fungal development and Arabidopsis transcriptional responses to *Botrytis cinerea* vary with time of inoculation

Some results from this chapter are published in Ingle *et al.,* (2015)

3.1 Introduction

The lesion size of *Arabidopsis thaliana* plants infected by *Botrytis cinerea* are significantly reduced following inoculation at subjective dawn compared to subjective night, suggesting time of inoculation influences infection development . It was further observed that the circadian clock of the host plant governs these differences as this pattern persists under free-running conditions and is abolished in dysfunctional circadian clock mutants. This indicates plants inoculated at subjective dawn react faster to *B. cinerea* attack and have a stronger defence response than those inoculated at subjective night, however mechanisms underlying the altered resistance levels remain unclear. From here, the pivotal question is what is how is the plant responding differently to inoculations that occur at subjective dawn compared to those that occur at subjective night?

Stomata play a key role in bacterial pathogen defence (Melotto *et al.,* 2006) and stomatal aperture has previously been shown to be under regulation of the circadian clock (Dodd *et al.,* 2005). Based on this evidence alone one hypothesis could be that the circadian regulation of stomatal aperture is affecting the ability of *B. cinerea* to enter the host and this could explain differences in resistance observed by Ingle *et al.,* (2015). However, it was observed *B. cinerea* do not use stomata as an entry point for infection.

Arabidopsis undergoes widespread transcriptional reprogramming during *B. cinerea* infection, with 9838 Arabidopsis genes (approximately one third of the genome) showing differential expression within 48 h of inoculation with *B. cinerea* 6 h after dawn (Zeitgeber time (ZT) 6) (under 16:8 light:dark (LD) conditions) (Windram *et al.,* 2012). Given this evidence, transcriptome-wide

changes were investigated by examining transcript levels using whole-genome microarrays following inoculation of Arabidopsis leaves with *B. cinerea* under constant light (LL) conditions at either subjective dawn (Circadian time (CT) 24) or subjective night (CT 42). Mock and inoculated leaf tissue was harvested 18 or 22 hours post inoculation (hpi) based on the Windram *et al.,* (2012) study where in-depth time course analysis following *B. cinerea* inoculation showed a large proportion of host transcriptional responses had occurred by 24 hpi. This chapter outlines the analysis steps taken to identify genes that had different expression patterns in response to *B. cinerea* inoculations that occur at subjective dawn compared to subjective night. These differentially expressed genes were mined for links to the circadian clock; they were assessed for rhythmicity under constant light conditions using Diurnal (Mockler *et al.,* 2007) and if any circadian clock core oscillator genes were bound to their upstream sequence (using publically available ChIP-seq datasets; Huang *et al.* (2012); Nakamichi *et al.* (2012), Liu *et al.* (2013); Kamioka *et al.,* (2016)). This analysis aimed to identify potential regulators of the time-of-day dependent resistance to *B. cinerea*.

3.2 Results

3.2.1 Circadian regulation of plant defence against *Botrytis cinerea* is not dependent on fungal strain used

The time-of-day dependent differences in resistance to *B. cinerea* observed in experiments performed by Ingle *et al.* (2015) used one *B. cinerea* strain (pepper). This section asks if these time-of-day differences in host resistance are dependent upon the fungal isolate used.

B. cinerea strains exhibit a high level of genetic variation; they show a higher level of genetic diversity than any other previously studied plant pathogen (Atwell *et al.,* 2015). Moreover, Canessa *et al.,* (2013) showed that under different lighting conditions various *B. cinerea* strains grow differently (Fig.3.1). Several *B. cinerea* strains were shown to be 'light responsive' in that they produce different development structures depending on whether it is light or dark. An example of this is strain BO5.10 that produces only sclerotia when kept under constant darkness (DD) and under LD conditions produces conidiophores and conidia (Canessa *et al.,* 2013) (Fig.3.1). However, some strains produce the same developmental structures independent of the lighting conditions, an example of this is strain T4 that can be seen in Fig.3.1. The *B. cinerea* pepper strain employed in the experiments by Ingle *et al.,* (2015) was similar to the T4 strain. The pepper strain showed no morphological differences whether it was grown under constant dark (DD) or LD conditions, employing the categorisation used by Canessa *et al.,* (2013) the pepper strain would fall into the "always conidia" grouping (Fig.3.1).

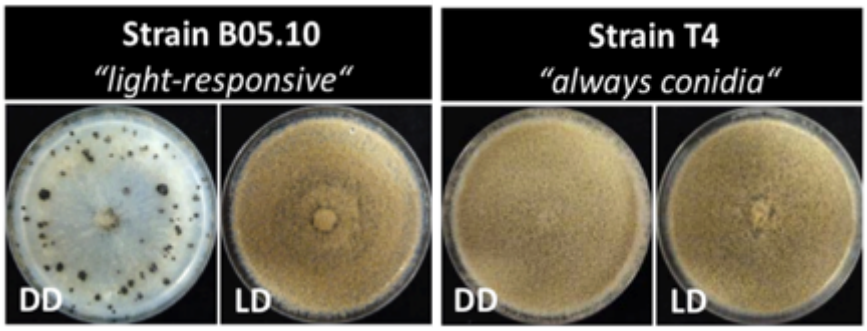


Figure 3.1 – Photomorphogenic developmental programs observed for two *B. cinerea* strains. Strains B05.10 and T4 incubated on solid complete medium for 14 days under LD (12:12 h light:dark) or DD (constant darkness). (Reproduced with permission from Canessa *et al.,* 2013).

Since the pepper strain employed in this thesis shows a very different light responsive phenotype to the BO5.10 strain. It could be that the pepper strain is also acting differently to the BO5.10 strain during the infection process and that the time-of-day dependent differences observed by Ingle *et al.,* (2015) with the pepper strain are not seen when inoculations are carried out using the BO5.10 strain. To further investigate this, plants were inoculated with two *B. cinerea* strains (Pepper or BO5.10) at subjective dawn (CT 24) or subjective night (CT 42) under LL conditions (Fig.3.2). Plants were transferred to LL conditions 24 h prior to inoculation and the remainder of the infection was left to progress under LL conditions.

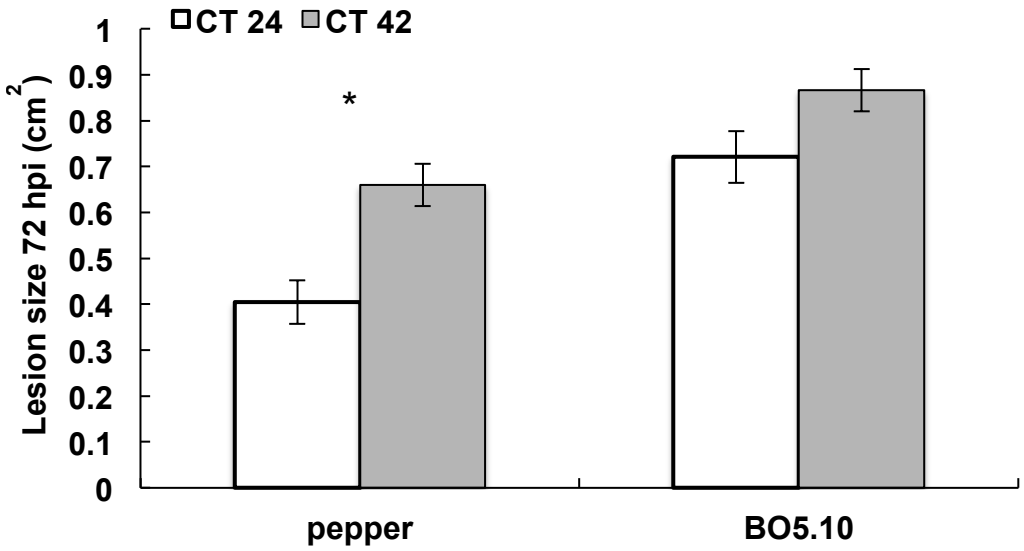


Figure 3.2 – Arabidopsis is more resistant to *B. cinerea* when inoculated at subjective dawn compared to when inoculated at subjective night, independent of the *B. cinerea* strain used. Detached leaves from four-week-old plants were inoculated with *B. cinerea* spores (pepper or B05.10 strain) at subjective dawn or subjective night under LL (CT 24 and CT 42) conditions and lesion area measured at 72 hpi. Leaves were transferred to LL 24 h prior to inoculation and the infection process took place under LL. Fungal spores were harvested from plates kept under constant dark conditions (DD) for 14 days. Data shown are representative of 2 independent experiments. Data shown are mean lesion sizes ± SEM on Col-0 (n ≥30 for each time of inoculation). Student's *t*-test was used to test whether lesion size was significantly different between leaves inoculated at dawn versus night; * indicates a *p*-value below 0.05.

Reduced lesion sizes in response to dawn inoculations suggested plants maintain a higher level of resistance to *B. cinerea* when inoculated at subjective dawn compared to when inoculated at subjective night, independent of whether they are inoculated with the *B. cinerea* strain pepper or BO5.10 (Fig.3.2). These results are in agreement with that of Hevia *et al.,* (2015). Hevia *et al.,* (2015) found that Col-0 plants inoculated using *B. cinerea* BO5.10 LL conditions exhibited a significant difference between inoculations that occurred at subjective dawn compared to subjective night, with plants showing greatest susceptibility when inoculated night.

3.2.2 Circadian regulation of stomatal opening does not play a role in time-of-day differences in Arabidopsis susceptibility to *B. cinerea*

Stomatal pores play various roles in plants, primarily, the intake of CO^2 for photosynthesis and allowing water loss from leaf tissue (Gudesblat *et al.,* 2009). However, possessing stomata in such close proximity to the environment can leave plants exposed to several environmental and biotic stresses. As such, plants have evolved to rapidly adjust stomatal aperture in response to changes in factors such as light, humidity and the presence of a pathogen (Gudesblat *et al.,* 2009). Upon detection of PAMPs from bacterial pathogens, plants will rapidly close their stomata to prevent colonization by pathogens (Melotto *et al.,* 2006).

Stomatal aperture has previously been shown to be under regulation of the circadian clock and aperture shows rhythmicity throughout the day (Dodd *et al.,* 2005). If *B. cinerea* enters through stomatal openings one hypothesis could be that the rhythmicity of stomatal aperture is affecting the ability of *B. cinerea* to enter the host and this could explain differences in resistance observed.

The role stomata have in the *B. cinerea* infection and colonization process is ambiguous, as unlike bacterial pathogens, this fungal pathogen secretes cell-wall degrading enzymes which break down the plant cell wall and allow the fungus to directly penetrate host tissue (Williamson *et al.,* 2007). However, open stomata and wounds have reportedly been exploited by *B. cinerea* when colonizing host tissue (Fourie and Holz, 1995).

To further investigate the role of plant stomata in the *B. cinerea* infection process, *B. cinerea* hyphae were observed growing in/on Arabidopsis leaves using trypan blue staining (Koch and Slusarenko, 1990) (Fig.3.3). From this it could be determined if *B. cinerea* hyphae had any preference for growing towards open stomata or entering open stomata.

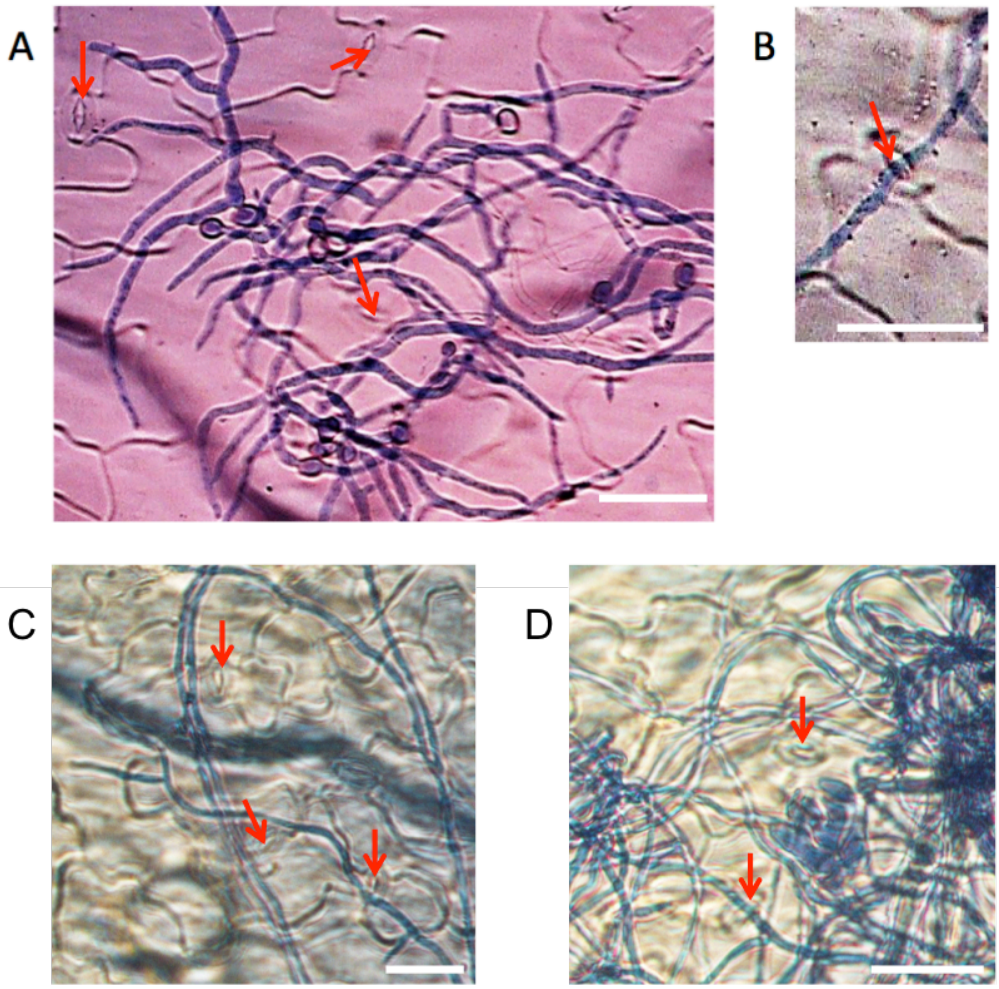


Figure 3.3 - Stomata are not a primary point of entry for *Botrytis cinerea* hyphae during infection of Arabidopsis. (A) Trypan blue-stained fungal hyphae on surface of Arabidopsis leaf at CT30, 12 h after inoculation (at subjective night (CT42)) with *B. cinerea* spores. (B) close-up image showing hypha crossing open stomata. (C) Trypan blue-stained fungal hyphae on surface of Arabidopsis leaf at CT44, 20 h after inoculation (at subjective dawn (CT24)) with *B. cinerea* spores (D) Trypan blue-stained fungal hyphae on surface of Arabidopsis leaf at CT40, 22 h after inoculation (at subjective night (CT42)) with *B. cinerea* spores. Red arrows indicate open stomata. Scale bars are representative of 80µm.fig4

Eighty leaves were inoculated with three *B. cinerea* droplets each at either subjective dawn (CT24) or subjective night (CT42), under constant light conditions. Five leaves were harvested at two h increments between 10-24 hpi and *B. cinerea* growth and stomatal opening patterns were observed, Fig.3.3 is a representative sample of these observations.

Stomata were not observed to be a primary point of entry for *B. cinerea* hyphae at any time point. There was no preferential growth of hyphae towards open stomata observed in the 80 samples (Fig.3.3 - A and C), suggesting *B. cinerea* does not preferentially use open stomata as an entry point for infection. This observation is in line with the results of Dugan & Blake (1989) who found no evidence of hyphal taxis towards stomata in the western larch. Furthermore, on several occasions hyphae were seen growing directly over open stomata (Fig.3.3 - B and D). These results suggest stomata are not vital sites of entry into Arabidopsis for *B. cinerea*; therefore time-of-day dependent variation in immunity to this pathogen is unlikely to be explained by the clock-governed regulation of stomatal aperture.

3.2.3 *B. cinerea* shows developmental differences in plants inoculated at subjective dawn compared to plants inoculated at subjective night

As previously outlined, *B. cinerea* growth is restricted following inoculation at subjective dawn more efficiently than when plants are inoculated at subjective night under LL and LD conditions . Trypan blue was used to visualize spore germination during infection of Arabidopsis leaves. Leaves were infected using droplet inoculation at subjective dawn (CT 24) or subjective night (CT 42), before being stained with trypan blue to visualize spore germination *in situ* (Fig.3.4). If a stage of fungal development could be identified as different between the two inoculation times it could give an indication as to which branch of host immunity is acting more effectively when plants are inoculated at subjective dawn compared to subjective night. For example, if *B. cinerea* spores inoculated at subjective dawn do not germinate as fast as those inoculated at subjective night then it is likely the plant is secreting more antimicrobial compounds inhibiting spore germination in the morning than later in the day.

A timeframe between 10-24 hpi was selected for these observations based on previous research that was carried out on samples inoculated at ZT 6 (Windram *et al.,* 2012). This showed the majority of host transcriptional responses to *B.*

cinerea do not occur prior to 10-12 hpi and by 24 hpi *B. cinerea* growth is entering a lag phase, colonization has been established and most transcriptional responses to infection have occurred (Windram *et al.,* 2012).

The experimental setup was similar to that previously outlined. Eighty leaves were inoculated with three *B. cinerea* droplets each at either subjective dawn (CT 24) or subjective night (CT 42), under LL conditions. Five leaves were then harvested every two h between 10-24 hpi. *B. cinerea* growth and developmental stages were observed using trypan blue staining followed by microscopy, images depicted in Fig.4 are a representative sample of these observations. Extra leaves were inoculated under the same experimental conditions and left to form lesions without invasive staining in order to confirm the time-of-day differences were maintained. In agreement with previous results, those leaves inoculated at subjective dawn showed enhanced resistance and smaller lesions compared to those inoculated at subjective night (data not shown).

Spore germination is an important stage in the early life cycle of *B. cinerea* and is defined by the conversion of conidia into germ tubes (Cotoras *et al.,* 2009). In agreement with Windram *et al.,* (2012), observations made at 10 hpi no spore germination could be seen (Fig.3.4 – A and B). By 12 hpi germination had initiated and there was a significant increase in hyphae and germ tube formation on hosts inoculated at night compared to those inoculated at dawn (Fig.3.4 – C and D). Cell death was observed between 14-22hpi, however this is not visible on the images due to different magnification planes. On average 500 spores/ 5 ul inoculum should be visualized in both images, however only 6 and 11 spores can be seen (C and D), this is likely due to high magnification not encompassing the full inoculum site, low germination rates due to the early TP or/and spores being visible in different field of view and only one field of view can be seen in each image.

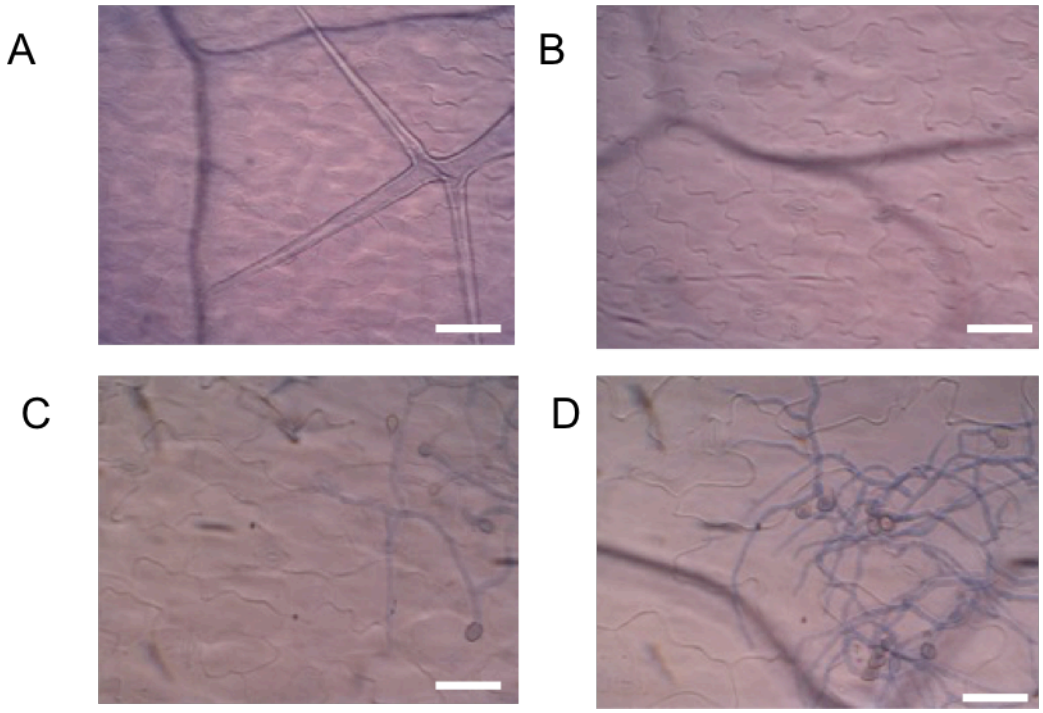


Figure 3.4 - Spore germination begins 12 hours post *Botrytis cinerea* inoculation of Arabidopsis leaves. Trypan blue-stained surface of Arabidopsis leaf under 20x magnification 10 h after inoculation at subjective dawn (CT24) (A) or at subjective night (CT42) (B) with *B. cinerea* spore inoculums. Trypan blue-stained surface of Arabidopsis leaf under 40x magnification 12 h after inoculation at subjective dawn (CT24) (C) or at subjective night (CT42) (D). 15 inoculation sites were observed for each time point and time of infection (either dawn or night) and no spore germination was seen at 10 hpi. Prior to germination *B. cinerea* spores do not take up the trypan blue stain. Scale bars are representative of 80μm.

Furthermore, between 16-18 hpi hyphal levels were higher on hosts inoculated at night compared to dawn (Fig.3.5). This could be due to one of two things; either the first germination stage of *B. cinerea* was delayed in inoculums that were applied at subjective dawn compared to subjective night, or the plant is suppressing the formation of hyphae more effectively when inoculated at dawn compared to night.

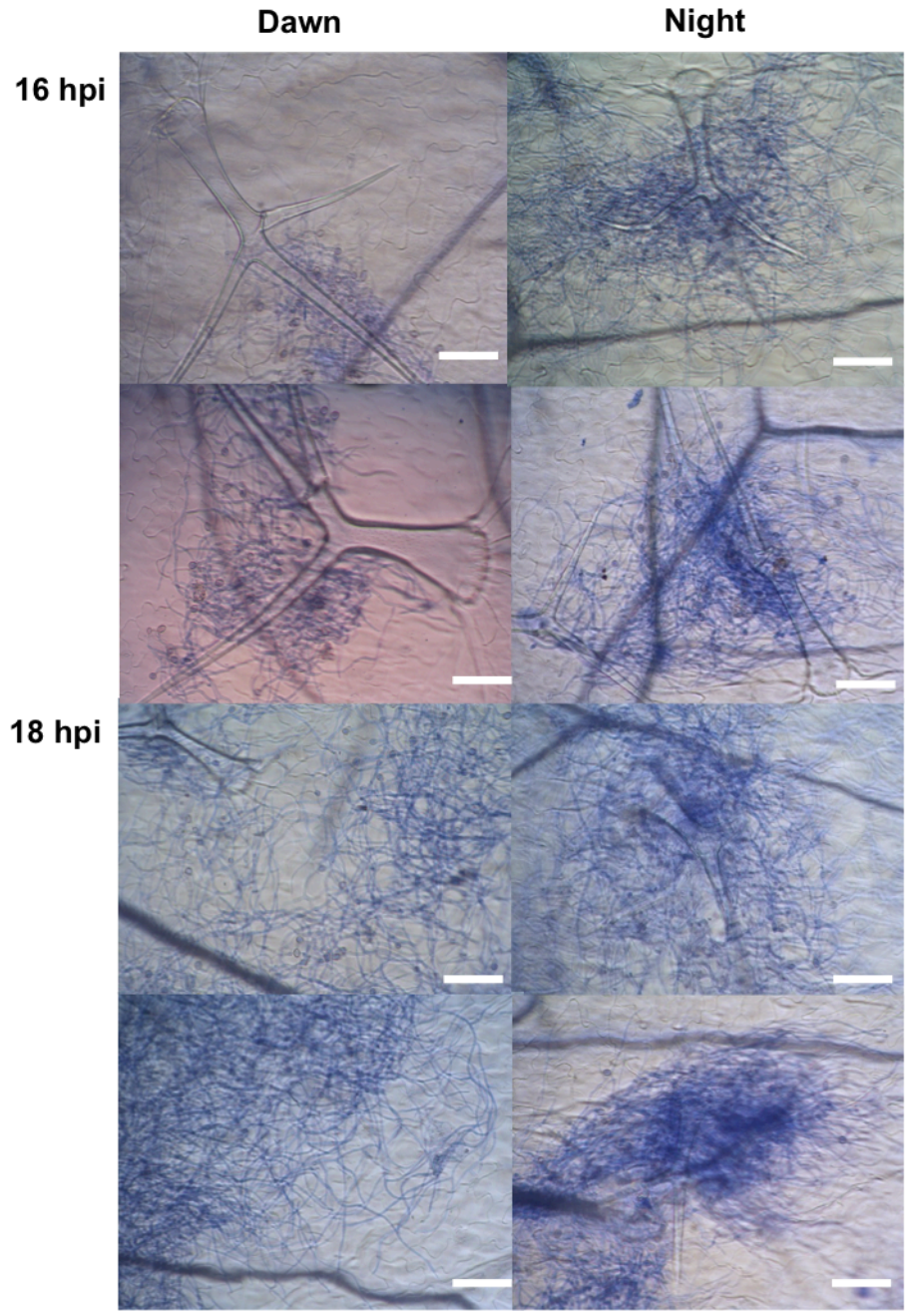


Figure 3.5 - *B. cinerea* hyphae formation is more developed on leaves inoculated at subjective night compared to subjective dawn at 16 and 18 hours post inoculation. Trypan blue-stained surface of Arabidopsis leaf under 20x magnification 16 h after inoculation at subjective dawn (CT24) (top left – 2 images) or at subjective night (CT42) (top right – 2 images) with *B. cinerea* spore inoculums. Or 18 h after inoculation at subjective dawn (CT24) (bottom left – 2 images) or at subjective night (CT42) (bottom right – 2 images). 15 inoculation sites were observed for each timepoint and time of infection (either dawn or night). Scale bars are representative of 80µm.

Botrytis was observed to form claw structures in the infected tissue. Very little is known about these thicker, claw-like structures, however they have

previously been reported to form between 18-20 hpi in inoculations that occurred at ZT 6 (Windram *et al.,* 2012) and have been linked to penetration of host tissue (Kunz *et al.,* 2006). Claw formation appears to be decreased in plants inoculated at subjective dawn compared to those inoculated at subjective night (Fig.3.6). The differences here may be due to the first germination stage of *B. cinerea* being delayed in inoculums that occurred at subjective dawn compared to subjective night hence all further stages are delayed or it may be due to a separate suppression by the plant. By 22-24 hpi the plant is fully colonised by *B. cinerea* and this made it difficult to make any definitive visual observations using trypan blue staining (Fig.3.6).

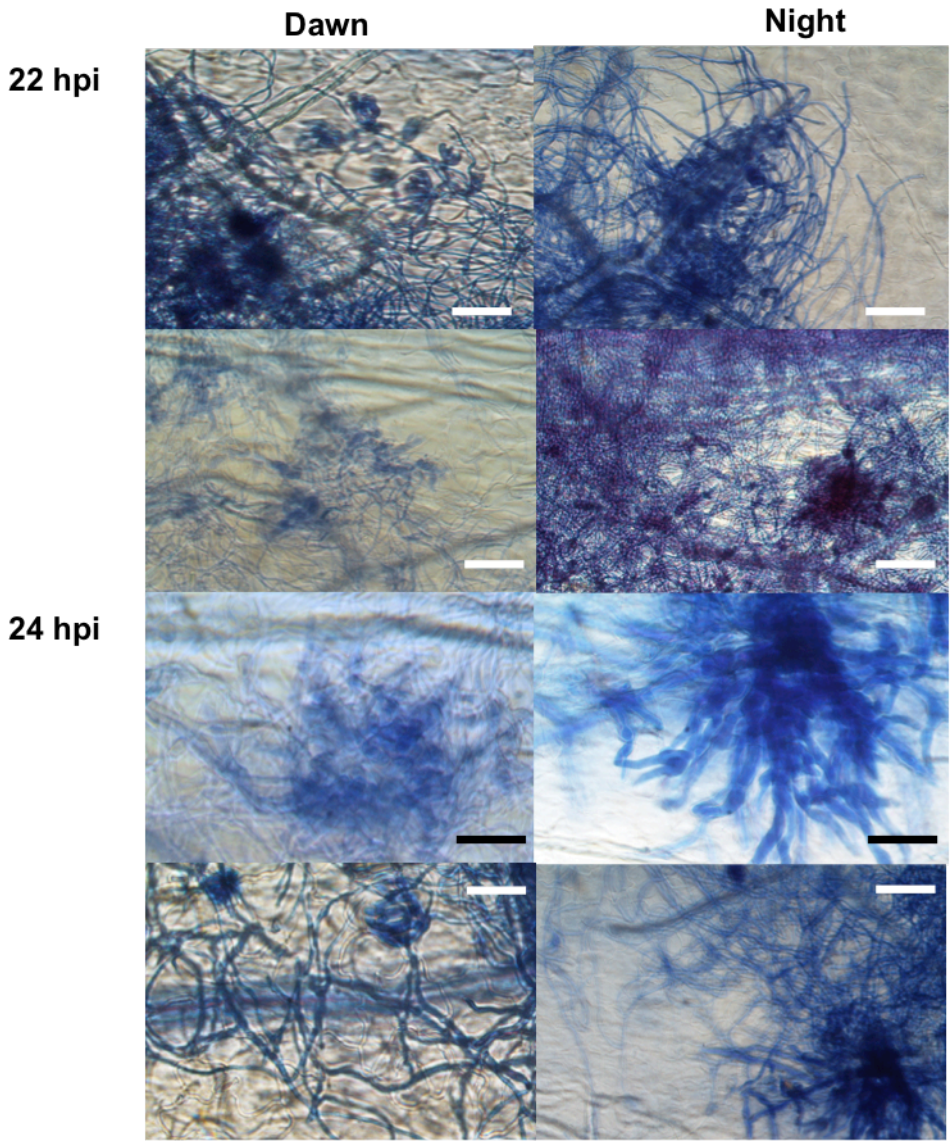


Figure 3.6 - *B. cinerea* 'claw' formation is faster on leaves inoculated at subjective night compared to subjective dawn at 22 and 24 hours post inoculation. Trypan blue-stained surface of Arabidopsis leaf under 20x magnification 22 h after inoculation at subjective dawn (CT24) (top left – 2 images) or at subjective night (CT42) (top right – 2 images) with *B. cinerea* spore inoculums. Or 24 h after inoculation at subjective dawn (CT24) (bottom left – 2 images) or at subjective night (CT42) (bottom right – 2 images). 15 inoculation sites were observed for each timepoint and time of infection (either dawn or night). Scale bars are representative of 80µm.

The first transcriptome changes observed in response to inoculations at ZT 6 (6 hours after dawn) were between 10 – 12 hpi, very few genes changed in expression prior to these time points (Windram *et al.,* 2012). First links with when first fungal hyphae development was observed (between 12-16 hpi when inoculations occur at subjective dawn and at 12 hpi when inoculations occur at

subjective night). The initial host gene expression changes therefore appear to occur just prior to the formation of hyphae. This initial change in gene expression is likely to impact the infection outcome. It could be that inoculations at subjective night progress more rapidly and the 'claw formation' occurs earlier as the genes differentially expressed 10 hpi in response to inoculations at ZT 6 are not differentially expressed in response to inoculations at night. Thus, as previously established plants inoculated at subjective dawn appear to mount a more effective defence response to *B. cinerea* than those inoculated at subjective night. It is likely plants achieve this by suppressing the very first stage of fungal development (germination and initial hyphal growth) more effectively when inoculated at dawn compared to night. Exactly how the plant is doing this is still unknown, however it appears to be linked to transcriptomic changes.

3.2.4 The Arabidopsis transcriptome responds differently after *B. cinerea* inoculation at subjective dawn compared to inoculation at subjective night

The first indication that the clock may impact transcriptional defence response came from the data generated by Windram *et al.,* (2012). Upon the detection of *B. cinerea* 9838 Arabidopsis genes (approximately one third of the genome) undergo differential expression within 48 h of inoculation with the fungus six h after dawn (ZT 6) (under 16:8 L:D conditions) (Windram *et al.* 2012). Furthermore, this study also observed that in uninfected time course samples, which were harvested every two h over a 48-hour period, 2404 genes displayed a circadian gene expression profile. Of these 2404 genes, over 60% were differentially expressed in response to *B. cinerea* inoculation (Windram *et al.,* 2012). As such, one potential explanation for the time-of-day dependent variation in susceptibility to *B. cinerea* in Arabidopsis is that the circadian clock is differentially regulating the speed and/or amplitude of the transcriptional defence response over the 24 h day.

To identify TFs responsible for the regulation of the Arabidopsis defence response against *B. cinerea* a gene regulatory network was created. TFs

differentially expressed (DETFs) in response to inoculation in the time series data generated by Windram *et al.,* (2012) were used as inputs into Causal Structure Identification (CSI) (CSI performed by Iulia Gherman, University of Warwick). DETFs from this data were exclusively used as inputs into CSI, as TFs are heavily responsible for the topology of networks. CSI was run with a marginal probability score of 0.026. This score was chosen as it generated a predicted gene regulatory network of 510 genes showing it to be highly stringent.

The full model was filtered to 510 TFs (Appendix 2), each were predicted to be involved with *B. cinerea* defence (as it was previously show to be DE in response to *B. cinerea* inoculation) and is also predicted have downstream connections in the network. TFs that lack downstream targets are not predicted to function as regulators of other genes in this defence network and were therefore omitted. Many of these genes have been previously shown to be related to circadian rhythms. 55% demonstrated rhythmic expression during constant light, 18% have been shown to be the direct targets of at least one of the clock proteins CCA1, TOC1, PRR5 or PRR7 (Table 3.1). From this data, it appears genes that were in the 510 TFs from the *B. cinerea* network model were significantly enriched for the binding of several circadian clock proteins (LHY, TOC1, PRR5 and PRR7) compared to the rest of the Arabidopsis genome.

Furthermore, a significantly greater proportion of the network model compared to the whole genome is rhythmic under constant light conditions (Mockler *et al.,* 2007). These factors combined suggest the circadian clock is directly regulating a large proportion of the Arabidopsis defence network. Moreover, it appears the clock is directly regulatory TFs in the network that are influencing downstream connections and are therefore vital for host transcriptional responses to *B. cinerea*, rather than individual downstream genes.

Table 3.1 – Key defence genes against *B. cinerea* are enriched for direct circadian clock gene binding. 510 genes were deemed to be key network genes regulating defence against *B. cinerea* by using CSI modelling. Direct binding targets of core circadian clock transcription factors: (TOC1 (Huang *et al.* 2012), PRR5 (Nakamichi *et al.* 2012), PRR7 (Liu *et al.* 2013) and CCA1 (Kamioka *et al*., 2016) were identified using ChIP-seq. These direct targets were then investigated for their presence in the 510-gene network or the whole Arabidopsis genome and enrichment tested for using hyper-geometric testing. Rhythmicity data from Diurnal (Diurnal datasets: LL12_LDHH, LL23_LDHH, LL_LLHC or LLHC with a correlation cut-off of 0.8) (Mockler *et al.,* 2007).

	Number of genes	**Number rhythmic in constant light**	**Direct targets of CCA1**	**Direct targets of TOC1**	**Direct targets of PRR5**	**Direct targets of PRR7**
Botrytis defence network genes	510	283 (55%)	25 (5%)	33 (6%)	26 (5%)	8 (2%)
Whole genome	27416	7196 (26%)	824 (3%)	867 (3%)	541 (2%)	113 (0.4%)
***P* value for enrichment**	-	3.47E-45	1.21E-02	9.51E-05	1.20E-05	1.26E-03

Given this information, it was hypothesised that the time-of-day dependent variation previously observed in plant responses to *B. cinerea* may be due to the circadian clock's regulation of the transcriptome.

Ingle *et al.,* (2015) observed transcriptome-wide changes by carrying out Nimblegen expression arrays following inoculation of Arabidopsis leaves under LL conditions at either subjective dawn (CT24) or night (CT42). Tissue was harvested 18 or 22 hpi based on the Windram *et al.,* 2012 study where in-depth time course analysis following *B. cinerea* infection showed a large proportion of host transcriptional responses occurred between 18 and 22 hpi. Ingle *et al.,* (2015) then validated the array results ensuring the infections had triggered the predicted transcriptional response in the host by comparing their expression data for 31 marker genes previously shown to be upregulated in response to *B. cinerea* (AbuQamar *et al.* 2006; Chen *et al.* 2010; Luo *et al.* 2010; Mengiste *et al.* 2003; Pre *et al.* 2008; Windram *et al.,* 2012). Of the 31, marker

genes, Ingle *et al.,* (2015) observed 27 were upregulated in the array they performed.

Once data was validated and normalised I analysed the data to find out how the Arabidopsis transcriptome was behaving differently in response to inoculations that occur at subjective dawn compared to those that occur at subjective night. For analysis methods see Chapter 2.

901 genes were differentially expressed (DEGs) in response to inoculation with *B. cinerea* at subjective dawn compared to subjective night (Ingle *et al.,* 2015 – supplementary dataset 2). All 901 genes were significantly differentially expressed in response to inoculation with *B. cinerea*, furthermore they were also shown to either respond differentially in leaves inoculated at subjective dawn compared to those inoculated at subjective night (Fig.3.7 – a) or/and they showed a significant difference in expression levels in leaves inoculated at subjective dawn compared to leaves inoculated at subjective night (Fig.3.7 - b).

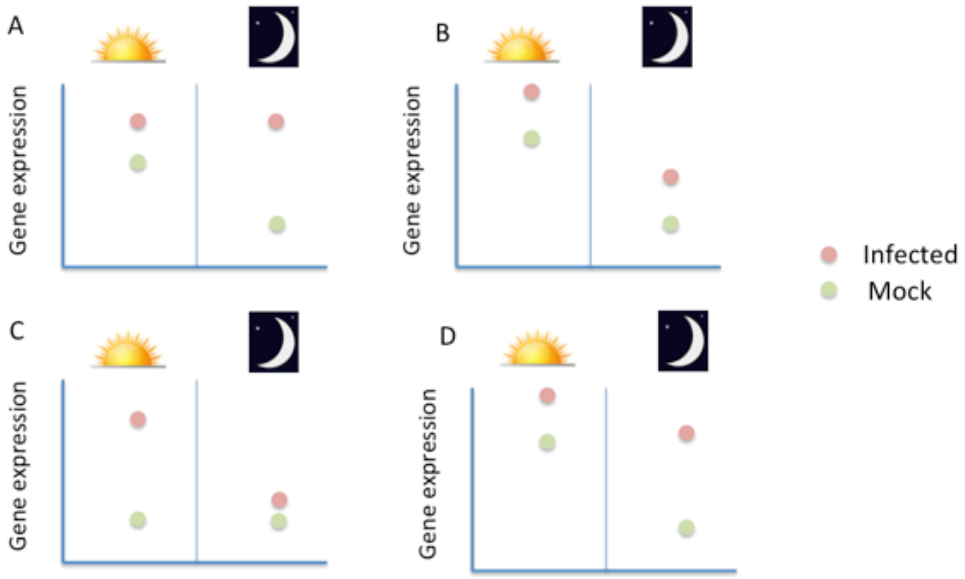


Figure 3.7 - Diagram illustrating selection of differentially expressed genes. Genes that showed a different fold change in expression in response to inoculation at subjective dawn and night but no subsequent difference in expression level (eg. A), genes with a similar fold change in response to infection at dawn or night but different levels of expression after infection (eg. B) and genes with different fold change and level of expression in response to infection at subjective dawn and night (eg. C and D) were selected. (Reproduced with permissions from: Ingle *et al.,* 2015).

To understand the biological processes occurring differently in response to inoculations at dawn compared to those at night, the 901 DEGs were grouped into eight non-overlapping groups based on their expression profiles in response to inoculation at either subjective dawn or subjective night (Ingle *et al.,* 2015, supplementary dataset 2), (Table 3.2).

Table 3.2 - Grouping of genes differentially expressed in response to infection and with a different expression level and/or induction after inoculation at subjective dawn compared to subjective night by 18 hpi or 22hpi.

Group	Biological significance	Number of genes
UPUP18	Genes upregulated in response to infection and with a higher expression level and/or induction after inoculation at dawn compared to night by 18 hpi	219
UPUP22	Genes upregulated in response to infection and with a higher expression level and/or induction after inoculation at dawn compared to night only at 22 hpi	44
DOWN DOWN18	Genes downregulated in response to infection and with a lower expression level and/or induction after inoculation at dawn compared to night by 18 hpi	257
DOWN DOWN22	Genes downregulated in response to infection and with a lower expression level and/or induction after inoculation at dawn compared to night only at 22 hpi	123
UPDOWN18	Genes upregulated in response to infection, but showed lower expression or reduced response to infection after inoculation at subjective dawn compared to night by 18 hpi	47
UPDOWN22	Genes upregulated in response to infection, but showed lower expression or reduced response to infection after inoculation at subjective dawn compared to night only at 22 hpi	98
DOWNUP18	Genes downregulated in response to infection but showed higher expression or response to infection after inoculation at subjective dawn compared to night by 18 hpi	53
DOWNUP22	Genes downregulated in response to infection but showed higher expression or response to infection after inoculation at subjective dawn compared to night at only at 22 hpi	48

The first four groups (UPUP18, UPUP22, DOWNDOWN18 and DOWNDOWN22) contain the majority (71%) of DEGs (Table 3.2). These groups seem to contain the genes whose expression pattern following infection is different between dawn and night inoculations. Genes within these groups appear to have an enhanced response in response to inoculations at dawn compared to night (whether its upregulation or downregulation). This enhanced response is likely to play a key role in the increased resistance in response to inoculations that occur at dawn compared to those that occur at night.

The subsequent 4 groups (UPDOWNs and DOWNUPs) display intuitive expression patterns, genes within these groups appear to have a reduced response to dawn compared to night inoculations (Table 3.2).

To gain insight into the biological processes occurring within the first four gene groups (whose expression seems enhanced in response to dawn inoculations), hence uncover the biological processes occurring differentially in response to inoculations occurring at subjective dawn compared to those occurring at subjective night, enrichment of Gene Ontology (GO) terms (Ashburner *et al.* 2000) were investigated in each of the four groups using BiNGO (Maere *et al.,* 2005) (Fig 3.8).

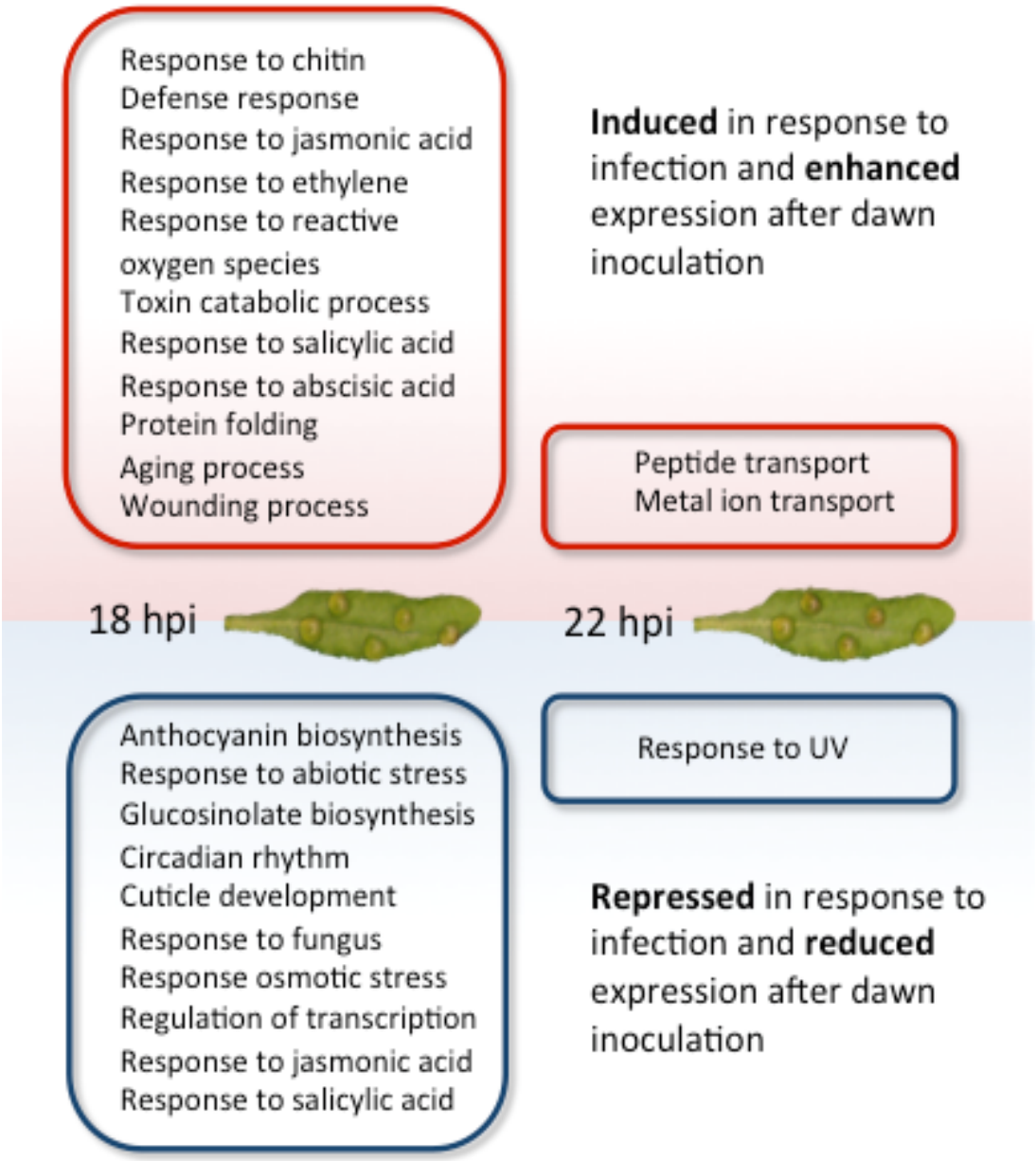


Figure 3.8 - Biological processes associated with the enhanced defence response at subjective dawn. The figure indicates selected GO terms enriched in genes whose response to infection is enhanced at subjective dawn compared to subjective night. (Reproduced with permissions from: Ingle *et al.,* 2015).

3.2.4.1 Confirming the responses

Most transcriptionally coordinated changes occur rapidly (by 18 hpi) and very few GO terms were enriched within groups of genes that change only at 22 hpi (Fig.3.8), this is similar to that observed by (Windram *et al.,* 2012), who

observed a small number of GO terms were associated with gene groups up-regulated later than 20 hpi.

The presence of these GO terms is very similar to the findings of (Windram *et al.,* 2012) in many ways. Windram *et al.,* (2012) found that genes upregulated at 16 hpi in response to *B. cinerea* inoculation were enriched for GO terms related to responses to JA, ET and oxidative stress. Observations here were in agreement with this, with the same responses overrepresented in genes upregulated at 18 hpi in response to inoculation at dawn. Processes repressed in response to *B. cinerea* infection in the Windram *et al.,* (2012) dataset included UV and UV related responses (such as flavonoid biosynthesis) and circadian rhythm. All were also repressed in response to inoculation at dawn in this dataset. The processes in Table 3.3 found to be enhanced or repressed in response to *B. cinerea* inoculation in Windram *et al,* (2012) were found to be more enhanced in response to inoculation at dawn compared to night or more repressed in response to inoculation at dawn compared to night. Suggesting processes vital to the defence response were more responsive to inoculations at dawn compared to night.

Table 3.3 – Comparing host transcriptional responses to *B. cinerea* between Windram *et al.,* (2012) and this dataset. DE – differentially expression. Grey – upregulated in response to infection and upregulated more in response to inoculation at dawn compared to night. White – downregulated in response to infection and downregulated more in response to inoculation at dawn compared to night.

GO term	Timing of first DE	
	DE in response to infection (Windram *et al.,* 2012)	DE in response to infection and time of inoculation (Ingle *et al*., 2015)
Response to JA	16 hpi	18 hpi
Response to ET	16 hpi	18 hpi
Response to oxidative stress	16 hpi	18 hpi
Response to UV and flavonoid biosynthesis	18-22 hpi	18-22 hpi
Circadian rhythm	24 hpi	18 hpi

The consistency between the two datasets confirmed that the defence response to *B. cinerea* follows a strictly timed, coordinated series of transcriptional events, many of which are differentially regulated in response to inoculations that occur at subjective dawn compared to those that occur at subjective night.

An interesting phenomenon is the presence of the GO terms 'response to SA' and 'response to JA' enriched in both gene groups that contain genes that are upregulated in response to infection and with a higher expression level and/or induction after inoculation at dawn compared to night (UPUP18) and those with genes that are downregulated in response to infection and with a lower expression level and/or induction after inoculation at dawn compared to night (DOWNDOWN18). There could be several explanations behind this: One possibility is that in some cases the GO term associations with those particular genes are tenuous. Another explanation could be that, given the multiple functions phytohormones play, gene groups represent different processes

governed by the same hormone, for example, genes involved in development and linked to JA for example could be in the DOWNDOWN18 group and those genes linked to JA and fungal defence could be in the UPUP18 group. To investigate this, the individual genes within each group and how these contribute to the GO term enrichment were investigated (Fig.3.9).

Looking at the individual genes associated with the GO term, 'Response to JA' there were 12 genes in the UPUP18 group and 15 genes in the DOWNDOWN18 group. To find the biological functions of these genes, all GO terms associated with each gene were retrieved. The genes within the response to JA GO term were also associated with other GO terms associated with phytohormone signalling, abiotic stress tolerance and wounding responses (Fig.3.9). The proportion of genes associated with each GO term between the UPUP18 and DOWNDOWN18 group differed (Fig.3.9).

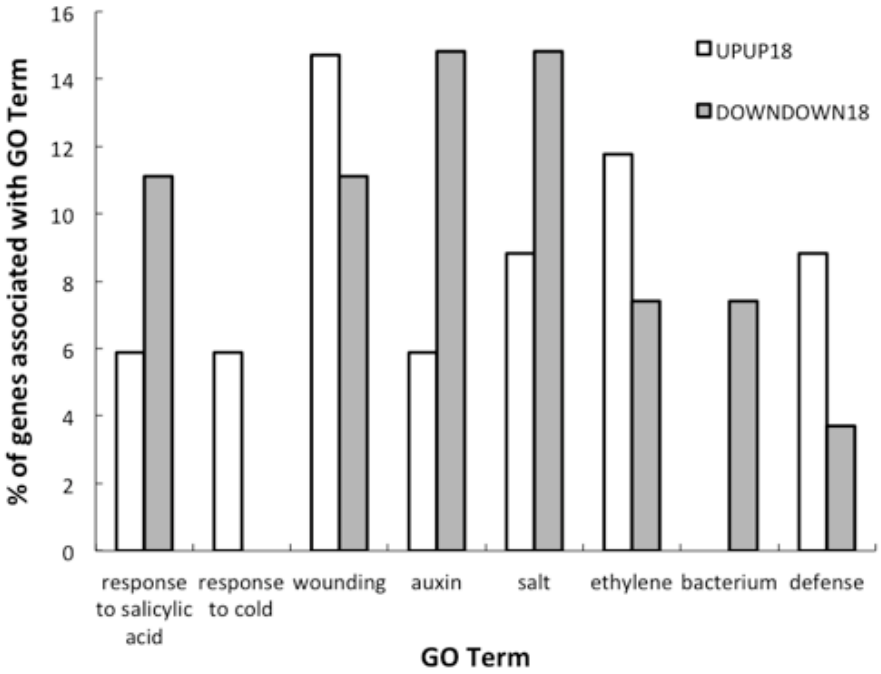


Figure 3.9 – Genes differentially up or downregulated in response to time of day and time of infection serve different roles within jasmonic acid responses. GO terms associated with individual genes found to be within the GO Term 'response to jasmonic acid' in the UPUP18 category or DOWNDOWN18 category. Gene numbers are plotted as a percentage of the total number of genes associated with the GO term, (UPUP18 gene n = 34, DOWNDOWN18 gene n= 27).

Genes linked to JA were also associated with the biotic defence hormones SA and ET, as well as auxin, in both the up and down regulated groups (Fig.3.9). Many of the genes linked to JA were also linked to SA (Fig.3.9). This is surprising given the antagonism and negative cross talk, which is thought to exist between the two pathways. A higher percentage of genes are linked to SA in the DOWNDOWN18 compared to the UPUP18 category. This may be because in the DOWNDOWN18 group there are more genes related to biotrophic defences which SA is known to play a pivotal role in and also the negative regulation of JA signalling which again, SA is known to play a key role in. As well as their antagonistic roles in pathogen defence, both SA and JA have been shown to be under circadian regulation, with actual JA and SA levels peaking out of sync with one another (Goodspeed *et al.,* 2012; Zhang *et al.,* 2013).

The crosstalk between JA and ET has been widely documented (reviewed in Yang *et al.,* 2015) and it has been shown that ET can act both agonistically and antagonistically with the JA pathway when working towards a common defence goal. There is also a lot of speculation around the role of ET in modulating the negative cross talk that exists between the JA and SA pathways (Yang *et al.,* 2015). It is therefore unsurprising that genes associated with JA are also associated with ET, genes such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *Related to AP2 6* (*RAP2.6*). The notable increase in the number of genes associated with ET in the UPUP18 compared to the DOWNDOWN18 group suggests in the case of *B. cinerea* defence the ET pathway plays more of a positive role (as supported by Chagué *et al.,* 2006). Again, both JA and ET levels have been measured and both have been shown to be under circadian regulation, again with ET peaking during the day under uninfected conditions (Thain *et al.,* 2004). This natural peaking may explain why so many ET genes are seen in the DOWNDOWN18 group, perhaps these transcripts were already decreasing in levels and the influence of infection just increased the rate of this.

There is staggering percentage (21%) of genes associated with both auxin and JA (Fig.3.9) and even more staggering, is the greatly increased number of genes

associated with both hormones in the DOWNDOWN18 compared to the UPUP18 category. Auxin has previously been shown to interact with both SA and JA during pathogen attack (Wang *et al.,* 2007; Woodward & Bartel, 2005). Although there is conflicting evidence if it widely accepted that auxin promotes resistance to biotrophic pathogens and promotes susceptibility to necrotrophic pathogens (Llorente *et al.,* 2008). This supports the obtained results in that more genes are downregulated in response to infection and time of day than are upregulated, showing auxin is unlikely mount a positive defence against *B. cinerea* attack. Auxin levels are under circadian regulation, peaking at midday and showing lowest levels during the night (Covington *et al.,* 2007). Even under mock conditions it is therefore predicted more auxin responsive genes would be found to be downregulated between the two time points, as one was during the day whereas the other was at night.

So within the GO term, 'Response to JA' there were 12 genes in the UPUP18 group, as well as being associated with many other hormones, several were associated with fungal defence, these included *PLANT DEFENSIN 1.2* (*PDF1.2*) and *MYB DOMAIN PROTEIN 108* (*MYB108*)). It follows that genes upregulated in response to infection and upregulated in response to infection at dawn more than infection at night would be related to fungal defence given the smaller lesion sizes observed after infections that occur at dawn compared to those that occur at night.

3.2.4.2 The GCC-box motif is overrepresented upstream of genes differentially regulated in response to inoculation at different times of day

To determine whether the promoters of genes responding differentially to infection at different times of the day were enriched for particular DNA sequences, MEME-LaB software (Brown *et al.* 2013) was employed. It was observed that genes upregulated in response to infection and with a higher expression level and/or induction after inoculation at dawn compared to night by 18 hpi (UPUP18) were enriched for the GCC-box promoter motif, suggesting possible co-regulation (Fig.3.10). It has been shown that genes containing the

same upstream motif sequences often bound by and regulated the same TFs (Vandepoele *et al.,* 2009).

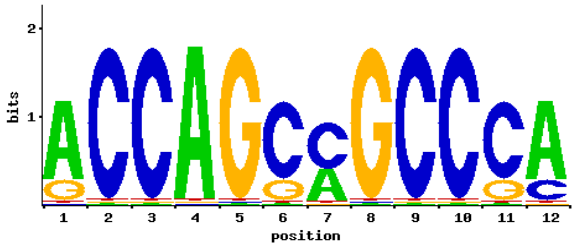


Figure 3.10 – GCC-box binding motif position weight matrix.

This motif is a known binding site for a number of AP2/Ethylene response factors (ERF) TFs and synergistic JA/ET signalling is known to converge on the GCC-box (Zarei *et al.,* 2011). This was found this upstream of five genes; *NAC DOMAIN CONTAINING PROTEIN 4* (*NAC004*), *PLANT DEFENSINS* (*PDF1.2b, PDF1.2, PDF1.2c*) and *SENESCENCE-RELATED GENE 3* (*SRG3*) (Fig.3.11). SRG3 has yet to be directly linked to *B. cinerea* defence, however, a strong link between senescence and defence to *B. cinerea* has been widely observed (Swartzberg *et al*. (2008); Windram *et al.* (2012)). Furthermore, a closely related gene (*SRG1*) has previously been shown to be significantly upregulated in response to *B. cinerea* infection (Expression Atlas: Kapushesky *et al.,* 2012). The three plant defensins found to contain the GCC-box motif have all previously been shown to play a positive role *B. cinerea* defence and they have also been found to accumulate in response to ET and JA (Penninckx *et al.,* 1998). Moreover, two more plant defensins (*PDF1.1* and *PDF1.3*) were also in the UPUP18 group and a motif similar to the GCC-box was identified in the upstream promoter sequence of *PDF1.3* (ATCATCAGCCCA).

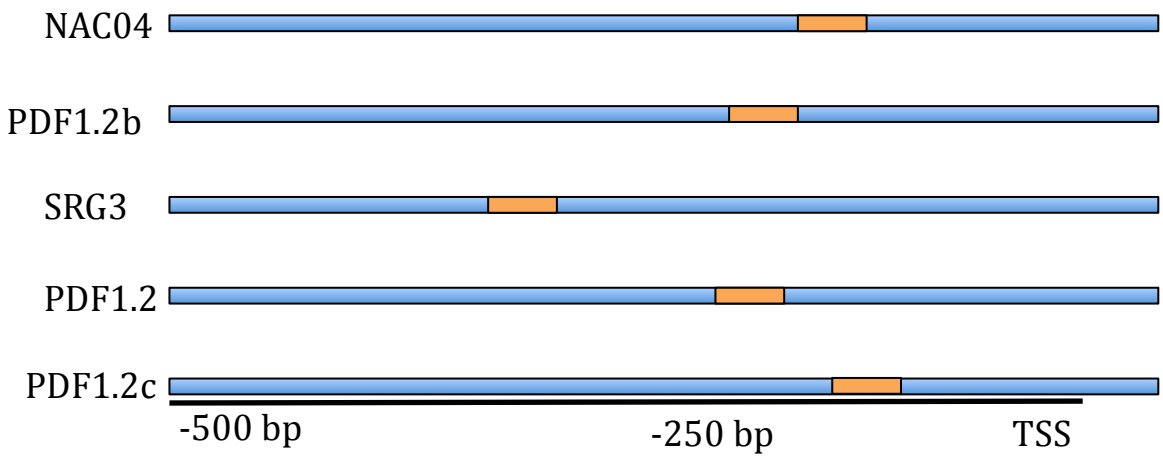


Figure 3.11 – Positions of GCC-box motif on forward strands of the 500bp promoter regions of five genes (*NAC04, PDF1.2b, DRG3, PDF1.2* & *PDF1.2c*) in the UPUP18 gene grouping. TSS – transcriptional start site.

Promoter sequence data was further mined for overrepresented motifs using recently released motif libraries from Weirauch *et al.,* (2014) and Franco-Zorilla *et al.,* (2014). Weirauch *et al.,* (2014) generated their library by first using computer inference methods (Cis-BP database (catalogue of inferred sequence binding preferences)) then confirming several of their results *in-vivo* using ChIP-seq. Whereas, Franco-Zorilla *et al.,* (2014) generated their library by confirming the target sequences of 63 plant TFs using protein-binding microarrays.

Within the Franco-Zorilla *et al.,* (2014) library the KAN1 (GAATAT) (Fig.3.12) binding motif was significantly overrepresented (after MTC $p = 0.036$) in the 200 bp upstream sequence region of genes in the UPUP18 group when using a Bonferonni MTC. Very little is known about the KAN1 motif, however, it has previously been shown to be bound by the GARP family of TFs including the transcriptional repressor, *KAN1* (*KANADI 1*) and *GLK1* (*GOLDEN2-LIKE 1*) (Franco-Zorilla *et al.,* 2014). Neither of these genes have been previously linked to defence, however, both display circadian expression patterns under long day conditions (Diurnal: Mockler *et al.,* 2007). Furthermore, *GLK2* (*GOLDEN2-LIKE 2*) (the redundant partner of *GLK1*) is repressed by CCA1/LHY (Kamioka *et al.,* 2016). Other members of the GARP family could also be binding to the KAN1 motif, there are at least 50 genes known to encode GARP proteins (Yanagisawa, 2013). One such protein is Phytoclock 1 (PCL1), which has previously been

shown to be vital in generating circadian oscillations (Onai *et al*., 2005).

Eight genes in the UPUP18 grouping contained the KAN1 motif in promoter sequences. Four of these genes were unnamed; as such to understand biological functions associated with these genes the individual GO Terms associated with each individual gene were investigated. One of the unnamed genes, AT1G71520, presumably plays some role in the defence response based on its associated with the GO Terms 'chitin response' and 'ethylene signalling'. Another unnamed gene, AT5G58390, is likely to play a role in the oxidative stress response given that seven of the nine GO terms associated with this gene were related to oxidative stress response. *RAP2.9* (*RELATED TO AP2 9*) is an ERF TF and was also found to have a KAN1 motif in its upstream sequence.

The KAN1 TF, which binds to the KAN1 motif, has been shown to repress the auxin-signalling pathway (Merelo *et al.,* 2013). Moreover, KAN1 has also been shown to regulate genes involved in a wide range of hormonal responses, including those that are ET, JA and ABA responsive (Merelo *et al.,* 2013). ChIP-seq analysis revealed KAN1 directly binds upstream of many genes involved in JA signalling (including *JAZ6, TPL* and *JMT*) and JA biosynthesis (*AOC1*), KAN1 also bound upstream of many genes related to ethylene processes (Merelo *et al.,* 2013). Moreover, KAN1 was also found to bind to the downstream regions of many genes related to JA biosynthesis (*AOC1* and *AOC3*), ethylene biosynthesis (*ACO2*) and signalling (*EIL1, ETR1* and *ERF4*) (Merelo *et al.,* 2013). Given the wide range of genes (*n* = 3151) found to be bound by KAN1 in ChIP-seq analysis it is likely this TF has a diverse range of functions, of which ET and JA signalling and biosynthesis appear to be amongst these (Merelo *et al.,* 2013).

Given that eight genes with the KAN1 motif in their upstream regions are upregulated more in response to inoculations that occur at dawn compared to night it could be that the KAN1 TF is binding these regions and playing a role in mediating the defence response differently in response to inoculations at dawn compared to night. However, to confirm this further work will need to be performed.

Both the GCC-Box and KAN1 binding motifs were significantly overrepresented upstream of several key defence genes that were upregulated in response to infection and had a higher expression level and/or induction after inoculation at dawn compared to night by 18 hpi. The discovery of these binding sites (GCC-Box and KAN1) suggest there could be a handful of TFs (such as KAN1) regulating genes and leading to coordinated rapid response to inoculations that occur at dawn but not those that occur at night. Genes that had the GCC-box or KAN1 binding motifs in their promoters were often found to play roles in the JA or/and ET signalling pathways (or example, the Plant Defensins are key JA and ET responsive genes), both hormones are vital for defence against *B. cinerea*. The roles JA and ET responsive pathways play in time-of-day dependent immunity will therefore be further explored.

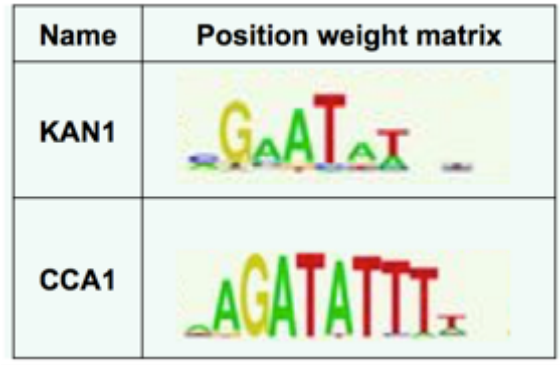


Figure 3.12 – Position weight matrices of KAN1 and CCA1 binding motifs. Adapted with permissions from Franco-Zorilla *et al.* (2014).

Using this method, it was also seen the CCA1 binding motif (AGATATTT) (Fig.3.12) (Franco-Zorilla *et al.,* 2014) was significantly overrepresented (after MTC $p = 0.047$) in the 200 bp upstream sequences of genes in the DOWNDOWN18 group, when a Bonferonni multiple testing correction (MTC) was applied. This group consists of genes that are downregulated in response to infection more if the inoculation takes place at dawn compared to night. 12 genes from this group had the CCA1 motif in their upstream promoter sequences. This further supports the hypothesis that the circadian clock is regulating the defence response to *B. cinerea* by directly binding to the promoters of key regulators of the *B. cinerea* defence pathway. It could be that genes from the DD18 group with the CCA1 binding motif in their promoter

regions are key regulators of *B. cinerea* defence. Two potential regulators of the defence response are discussed and other genes from this group with the CCA1 binding motif upstream are largely unknown or involved in chloroplast/photosynthetic functions.

A gene within this group is *PRR9*, a key circadian oscillator gene. CCA1 binds to the *PRR9* promoter region and positively regulates expression (Farré *et al.,* 2005). This could potentially be regulating further downstream effects in the defence response pathway against *B. cinerea*.

Moreover, the CCA1 binding motif was also found upstream of a well-known abiotic stress responsive gene within the DD18 group; *DREB1A* (*DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1A*). Abiotic stress responsive genes have previously been shown to contain the CCA1 binding motif in their promoter regions and the clock has been shown to have a major input into the abiotic stress response pathways (this has been previously discovered and discussed in Dong *et al.,* 2011). *DREB1A* is involved in cold and dehydration responses in Arabidopsis (Liu *et al.,* 1998) and Tobacco (Kasuga *et al.,* 2003). It could be that as well as abiotic stress tolerance; *DREB1A* also plays a regulatory role in biotic stress responses. Or it could be that as a positive key regulator of abiotic stress responses, *DREB1A* is downregulated in response to inoculation as part of the antagonism that exists between the abiotic and biotic defence pathways (Yasuda *et al.,* 2008). If the latter is the case, then it could be that the clock is directly modulating the antagonism between abiotic tolerance pathways and biotic stress responses.

3.2.4.3 Key defence-related transcription factors show differential responses to *B. cinerea* inoculation occurring at different times of day

The Arabidopsis genome is thought to encode approximately 2000 TFs, (Pruneda-Paz *et al*., 2014) which can be grouped into approximately 50 families, many of these families (such as WRKYs and ERFs) have previously shown to be vital for host defence responses against *B. cinerea* (Windram *et al.,*

2012). Of the 901 DEGs in response to infection and responding differently in response to inoculation at dawn compared to inoculation at night, 99 were TFs . Several individual TFs in this group of 99 have been shown to be crucial in host responses against *B. cinerea*. For example, *MYB108* and *ERF6* were both in the UPUP18 grouping and when reduced in expression of either TF, plants have shown increased susceptibility to *B. cinerea* (Mengiste *et al.* 2003; Moffat *et al.* 2012).

Furthermore, the group of 99 DETFs were also significantly enriched for rhythmicity under constant light conditions and was also significantly enriched for genes that are direct targets of several core circadian clock oscillators (TOC1, PRR5, PRR7 & CCA1) compared to the full Arabidopsis genome. Over 70% of these 99 DE TFs were shown to exhibit circadian expression profiles under LL conditions compared to the 26% rhythmicity observed in the whole genome (Diurnal: Mockler *et al.,* 2007) (Table 3.4). Moreover, 523 (58%) of the 901 DEGs exhibited circadian expression profiles under LL conditions, while this is much greater than the rhythmicity observed in the whole genome DETFs show 13% greater rhythmicity than the DEGs (Table 3.4). The circadian oscillators also appear to bind directly to more of the 99 TFs than all the 901 DEGs, for example, CCA1 was found to be directly binding 71% of the 99 TFs and only 14% of the DEGs (Table 3.4). This again points to the clock having a direct role in regulating the *B. cinerea* defence response by acting directly on transcriptional regulators in the pathway rather than further downstream genes.

Table 3.4 – Transcription factors differentially expressed in response to inoculation at subjective dawn or subjective night are significantly enriched for circadian clock binding compared to the rest of the genome. 99 DETFs and 901 DEGs identified from transcriptomic analysis as DE in response to inoculation at time of inoculation were tested for circadian expression patterns in LL (data from Diurnal (Diurnal datasets: LL12_LDHH, LL23_LDHH, LL_LLHC or LLHC with a correlation cut-off of 0.8) (Mockler *et al.,* 2007)). Binding of core circadian clock transcription factors to these genes was investigated using ChIP-seq datasets: (TOC1 (Huang *et al.* 2012), PRR5 (Nakamichi *et al.* 2012), PRR7 (Liu *et al.* 2013) and CCA1 (Kamioka *et al*., 2016). Enrichment for binding or rhythmicity in the 99 DETFs compared to the whole Arabidopsis genome was investigated using hyper-geometric testing.

	Number of genes	Number rhythmic in constant light	Direct targets of CCA1	Direct targets of TOC1	Direct targets of PRR5	Direct targets of PRR7
DETFs	99	70 (71%)	70 (71%)	11 (11%)	9 (9%)	11 (11%)
Whole genome	27416	7196 (26%)	824 (3%)	867 (3%)	541 (2%)	113 (0.4%)
***P* value for enrichment**	-	2.73E-20	6.03E-84	2.89E-04	1.52E-04	3.30E-13
DEGs	901	523 (58%)	127 (14%)	41 (5%)	29 (3%)	13 (1%)

TFs within the group of 99 showed different expression profiles in response to inoculations and time of day, the majority (58) of the 99TFs were in the DOWNDOWN18 (n = 35) and UPUP18 (n = 23) groupings, suggesting a coordinated response occurring rapidly when inoculated dawn. The remaining 41 TFs fell into all groupings: UPUP22 (n = 5), DOWNDOWN22 (n = 10), UPDOWN18 (n = 2), UPDOWN22 (n = 10), DOWNUP18 (n = 5) and DOWNUP22 (n = 9).

The groupings are made up of TFs with different mechanistic patterns, these TFs can be grouped into three groups; changes in expression level only, change in fold change only or changes in both expression level and fold change (See Fig.3.7). Differential basal expression of key defence TFs at dawn compared to night with no difference in fold induction were observed (Referred to as 'expression-level' in Table 3.5) (Fig.3.7– B). Expression profiles such as this suggest that plants are 'primed' for inoculations that occur at dawn compared to those that occur at night, as expression of some key defence TFs is higher at this time with or without inoculation.

Another distinct expression pattern observed is the differential induction or repression of key defence TFs in response to inoculation at subjective dawn versus subjective night leading to different expression levels (Fig.3.7– C, Table 3.5). These genes are an example of the how the circadian clock is mediating the plant defence system by gating specific genes. *BT2* is an example of this situation in that, inoculations that occur at subjective dawn have a greater induction and lead to higher expression of the TF than those inoculations that occur at subjective night. Genes such as *BT2* in this group have a higher expression level and induction response when inoculated at dawn compared to night; it is therefore likely these have a functional role in the differential defence response.

The final expression profile exhibited by these TFs was differential fold induction/repression in response to inoculation at subjective dawn or night but the resulting expression levels were not different. Referred to as 'fold-change' in Table 3.5). The nature of this differential regulation suggests the TFs in this group are very tightly synchronized and/or they potentially have a 'capped' expression level in that they cannot exceed a particular level.

Table 3.5 – Expression groupings of 99 Transcription Factors shown to be differentially expressed in response to time of inoculation and in response to infection. 99 DETFs as DE in response to inoculation at time of inoculation were tested for circadian expression patterns in LL (data from Diurnal (Diurnal datasets: LL12_LDHH, LL23_LDHH, LL_LLHC or LLHC with a correlation cut-off of 0.8) (Mockler *et al.,* 2007)). Binding of core circadian clock transcription factors to these genes was investigated using ChIP-seq datasets: (TOC1 (Huang *et al.* 2012), PRR5 (Nakamichi *et al.* 2012), PRR7 (Liu *et al.* 2013) and CCA1 (Kamioka *et al*., 2016).

AGI	Gene name	Expression profile	Group	Rhythmic (LL)	Clock TF direct targets			
					TOC1	PRR5	PRR7	CCA1
AT1G01060	LHY	both	DOWNUP18	yes	yes	yes	yes	yes
AT2G43010	PIF4	expression level	DOWNDOWN18	yes	yes	yes	yes	
AT2G31380	STH	fold change	DOWNDOWN22	yes	yes	yes	yes	
AT5G37260	RVE2	fold change	DOWNUP18	yes			yes	
AT2G46790	PRR9	both	DOWNDOWN18	yes	yes	yes	yes	
AT2G46830	CCA1	both	DOWNUP18	yes	yes	yes	yes	yes
AT2G22840	GRF1	expression level	DOWNDOWN18	yes	yes			
AT5G57660	COL5	expression level	DOWNDOWN18	yes		yes		
AT4G01250	WRKY22	expression level	UPUP18	yes	yes			
AT4G25480	DREB1A	fold change	DOWNDOWN18	yes		yes	yes	
AT4G27310	BBX28	fold change	UPDOWN22	yes	yes		yes	
AT3G47500	CDF3	both	DOWNDOWN18	yes		yes		
AT5G08330	TCP21	both	DOWNDOWN18	yes				yes
AT3G48360	BT2	both	UPUP18	yes			yes	
AT5G43270	SPL2	expression level	DOWNDOWN18	yes				
AT5G62470	MYB96	expression level	DOWNDOWN18	yes				
AT2G21530	AT2G21530	expression level	DOWNDOWN22	yes				
AT3G02380	COL2	expression level	DOWNUP18	yes				yes
AT2G22770	NAI1	expression level	UPUP18	yes				
AT1G71520	AT1G71520	expression level	UPUP18	yes				
AT1G28360	ERF12	expression level	UPUP22	yes	yes			
AT4G01060	CPL3	fold change	DOWNDOWN18	yes				
AT2G21320	BBX18	fold change	DOWNDOWN18	yes				
AT3G63030	MBD4	fold change	DOWNDOWN22	yes				
AT5G62430	CDF1	fold change	DOWNDOWN22	yes				yes
AT5G13730	SIG4	fold change	DOWNUP22	yes			yes	
AT3G12910	AT3G12910	fold change	UPDOWN22	yes				
AT3G23240	ERF1	fold change	UPDOWN22	yes				
AT3G21150	BBX32	fold change	UPDOWN22	yes				
AT4G06746	RAP2.9	fold change	UPUP18	yes				yes
AT3G50260	CEJ1	fold change	UPUP18	yes				
AT2G40350	AT2G40350	fold change	UPUP22	yes	yes			yes
AT1G71030	MYBL2	fold change	UPDOWN22	yes				yes
AT2G17040	NAC036	both	DOWNDOWN18	yes				
AT1G18710	MYB47	both	DOWNDOWN18	yes				
AT5G11590	TINY2	both	DOWNDOWN18	yes				
AT4G40060	HB16	both	DOWNDOWN18	yes				
AT4G17460	HAT1	both	DOWNDOWN18	yes				
AT1G51140	AKS1	both	DOWNDOWN18	yes				
AT4G32980	ATH1	both	DOWNDOWN22	yes				
AT4G17490	ERF6	both	UPUP18	yes				
AT3G06490	MYB108	both	UPUP18	yes				
AT5G64750	ABR1	both	UPUP18	yes				
AT2G28550	RAP2.7	expression level	DOWNDOWN18	yes				
AT1G49560	AT1G49560	expression level	DOWNDOWN18	yes				
AT4G36870	BLH2	expression level	DOWNDOWN18	yes				
AT5G60100	PRR3	expression level	DOWNDOWN18	yes				yes
AT5G25190	ESE3	expression level	DOWNDOWN18	yes				
AT1G73870	BBX16	expression level	DOWNUP22	yes				
AT1G02230	NAC004	expression level	UPUP18	yes				
AT1G61215	BRD4	expression level	UPUP18	yes				
AT1G66600	ABO3	expression level	UPUP18	yes				
AT5G40340	AT5G40340	expression level	UPDOWN22	yes				
AT5G60910	AGL8	expression level	DOWNUP22	yes				
AT4G09820	TT8	fold change	DOWNDOWN22	yes				

AGI	Gene name	Expression profile	Group	Rhythmic (LL)	Clock TF direct targets			
					TOC1	PRR5	PRR7	CCA1
AT4G38960	BBX19	fold change	DOWNDOWN22	yes				
AT2G30424	TCL2	fold change	DOWNUP22	yes				
AT4G14560	IAA1	fold change	DOWNUP22	yes				
AT5G61270	PIF7	fold change	UPUP22	yes				
AT3G23050	IAA7	fold change	DOWNUP22	yes				
AT3G58120	BZIP61	fold change	DOWNUP22	yes				
AT5G15310	MYB16	both	DOWNDOWN18	yes				
AT3G09600	LCL5	both	DOWNUP18	yes		yes		
AT5G65080	MAF5	both	DOWNDOWN22	yes				yes
AT2G18300	HBI1	both	DOWNDOWN18	yes				yes
AT5G62165	AGL42	both	UPUP18	yes				yes
AT5G43410	ERF96	both	UPUP18	yes				yes
AT5G52020	AT5G52020	both	UPUP18	yes				yes
AT5G43290	WRKY49	both	DOWNUP22	yes				yes
AT1G73830	BEE3	both	UPUP22	yes				yes
AT5G22570	WRKY38	both	UPDOWN18	yes				yes
AT1G46480	WOX4	expression level	DOWNDOWN18					
AT3G17609	HYH	expression level	UPUP18				yes	
AT5G56840	AT5G56840	expression level	UPUP18					
AT2G39900	WLIM2A	expression level	DOWNDOWN18					
AT1G01250	AT1G01250	fold change	DOWNDOWN18					
AT2G25820	ESE2	fold change	DOWNDOWN18					
AT1G74080	MYB122	fold change	UPDOWN22					
AT1G07050	AT1G07050	both	DOWNDOWN18					
AT3G24520	HSFC1	both	DOWNDOWN18					
AT5G07690	MYB29	both	DOWNDOWN18					
AT2G25000	WRKY60	both	DOWNDOWN22					
AT2G47520	HRE2	both	UPUP18					
AT3G04420	NAC048	both	UPUP18		yes			
AT2G18328	RL4	both	DOWNUP22					
AT2G18380	GATA20	expression level	DOWNDOWN22					
AT1G08290	WIP3	expression level	UPUP18					
AT4G26120	AT4G26120	expression level	UPUP18					
AT4G22770	AHL2	fold change	DOWNDOWN18					
AT4G32280	IAA29	fold change	DOWNDOWN18					
AT5G01900	WRKY62	fold change	UPDOWN18					
AT2G32030	AT2G32030	fold change	UPDOWN22					
AT5G48560	CIB2	fold change	UPUP18					
AT1G68150	WRKY9	fold change	UPUP22					
AT1G56650	PAP1	both	DOWNDOWN18					
AT5G26170	WRKY50	both	DOWNDOWN18					
AT2G47270	UPB1	both	UPDOWN22					
AT1G43160	RAP2.6	both	UPUP18					

Individuals within this group of 99 TFs are linking the circadian clock to the defence network and signalling within the defence network. The aim of this analysis was to identify which TFs are most likely to be linking the circadian clock to the defence network. To identify such candidates a selection criterion was employed (Fig 3.13).

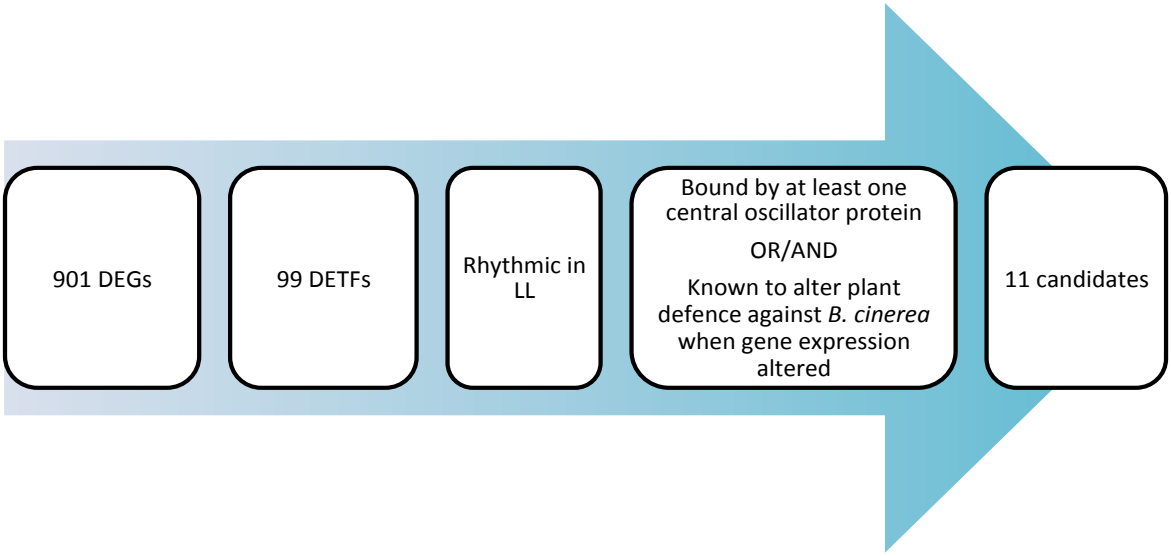


Figure 3.13 – Selection criteria to identify candidate transcription factors likely to be regulating time-of-day dependent plant immunity to *B. cinerea*. 901 differentially expressed genes (DEG) were identified; of these 99 were transcription factors (DETF). Those DETFs that were rhythmic in constant light (LL) were considered more likely to be under circadian regulation than those non-rhythmic so these were selected. Within this group of rhythmic DETFs those that were either bound by a central oscillator protein or/and caused a change in plant resistance levels to *B. cinerea* when gene expression was mutated were selected. This gave a total of 11 DETFs likely to be regulating time-of-day dependent plant immunity to *B. cinerea*.

11 of the 99 DETFs were identified as potential candidates for linking the clock to the defence network (Table 3.5). To confirm these candidates are linking the clock and the defence network, knockout and over-expressor lines of each gene should be screened for time of day dependent immunity to *B. cinerea* by inoculating it under LL conditions at subjective dawn (CT 24) and subjective night (CT 42) and LD conditions at subjective dawn (ZT 0) and subjective night (ZT 18).

Candidates (*STH, PIF4, RVE2, WRKY22, DREB1A, TCP21, BT2, COL2, ERF1, RAP2.9* and *MYB108)* displayed rhythmic expression patterns under LL and 9 of the 11 genes were directly bound by circadian clock genes. Only, *ERF1* and *MYB108* had no clock proteins bound to their sequences. The latter two genes were identified as candidates based on their phenotypes in *B. cinerea* screens. *ERF1* has previously been shown to confer resistance to *B. cinerea* when overexpressed (Berrocal-Lobo *et al.,* 2002) and when *MYB108* expression is

reduced plants exhibit increased susceptibility to *B. cinerea* (Mengiste *et al.* 2003). Example expression patterns of several of these candidates can be seen in Fig.3.14.

BT2 (*BTB and TAZ domain protein*) is potentially linking the circadian clock to the defence network; it is in the UPUP18 grouping, has a rhythmic expression pattern and is directly bound by PRR7. *BT2* has previously been shown to be under circadian regulation and has many roles in abiotic stress responses, including tolerance against cold stress (Mandadi *et al.,* 2009). Hormones have been shown to modulate *BT2* expression levels; MeJA and ABA positively and negatively regulate *BT2* expression levels, respectively (Mandadi *et al.,* 2009). It can be postulated that *BT2* acts in the antagonism between JA and ABA (Anderson *et al.,* 2004) and serves as a link between JA and the circadian regulation of biotic and abiotic stresses.

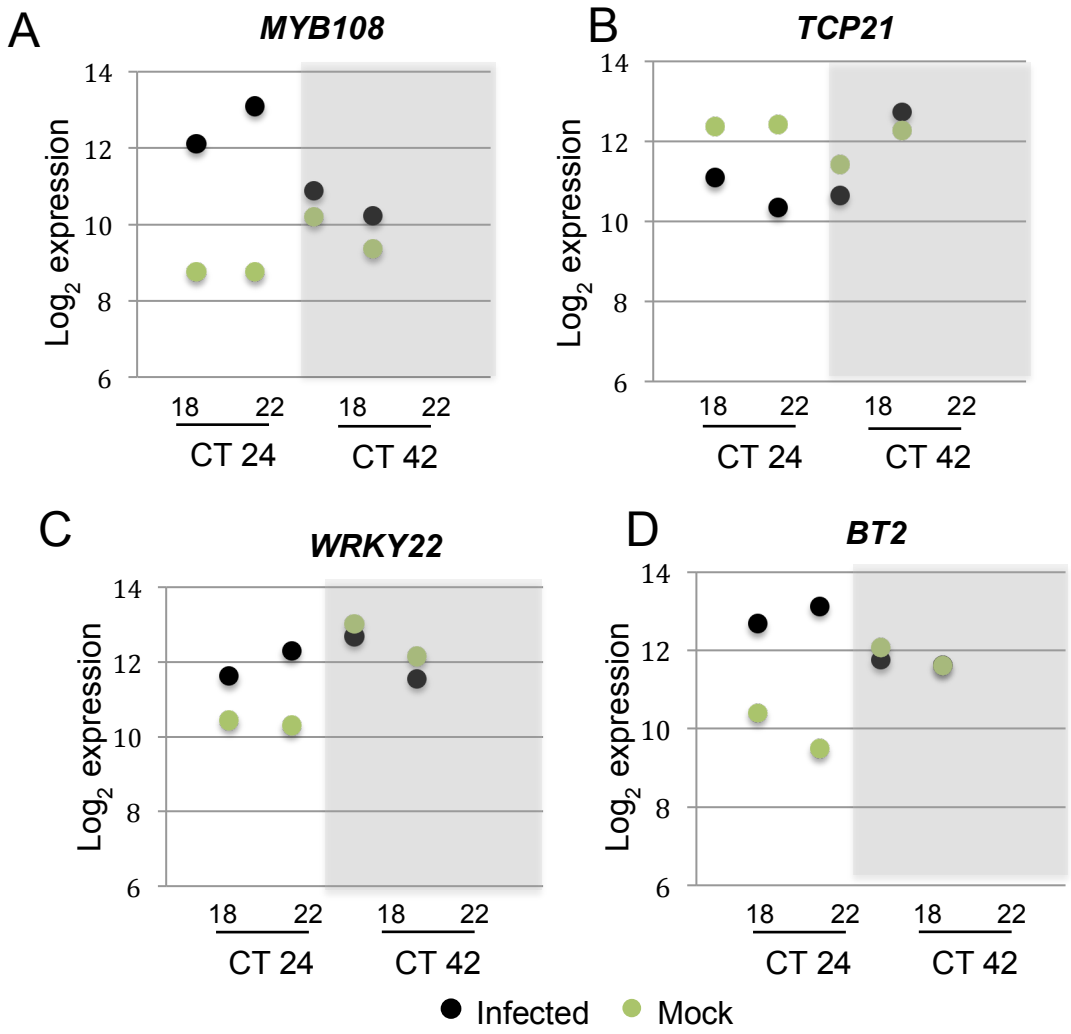


Figure 3.14 - Differential expression of transcription factor (TF) encoding genes in response to infection at subjective dawn or night under LL conditions. A) *MYB108* and D) *BT2* a higher expression level and induction response when inoculated at CT 24 (dawn) compared to CT 42 (night). TCP21 (B) has decreased expression level and is repressed more greatly when inoculated at CT 24 (dawn) compared to CT 42 (night). WRKY22 (C) is induced more in response to inoculations at CT 24 (dawn) compared to CT 42 (night), however the expression levels of WRKY22 are higher at night. Black circles are expression values after *B. cinerea* infection and green circles from mock-inoculated leaves. Data are from the microarray experiment.

3.2.5 Jasmonic acid levels are increased more in response to dawn compared to night inoculations

The role of JA in plant defence against necrotrophic pathogens is well documented (Thomma *et al.,* 1998) and given the evidence above it could be that this defence is at least partially governed by the circadian clock. The UPUP18 group of genes had significant enrichment of the GO term 'response to JA' showing this group of genes to play a key role in JA responses. Moreover, this gene group also showed significant overrepresentation of the GCC-box motif in their upstream sequences showing a coordinated biological function related to JA or ET responses within this gene group.

Given this the levels of JA in leaves inoculated or mock inoculated at dawn or night were measured. Plants were grown for 4 weeks under 16:8 LD conditions and then placed in LL for 24 h prior to inoculations. Levels of JA were measured in leaves every 2 h between 12 - 20 hpi following inoculation with mock or spore suspensions at either dawn or night under LL conditions. These hpi were selected as this timeframe was shown to have the most transcriptional changes occurring in response to *B. cinerea* inoculation (Windram *et al.,* 2012).

The protocol followed to measure JA levels is outlined in Forcat *et al.,* (2008), this method was validated by measuring JA levels in unwounded and mechanically wounded five-week-old Arabidopsis leaves. Values between the absolute and actual levels obtained in Forcat *et al.,* (2008) and our data in Fig.3.16 were highly similar. Actual levels of JA found in unwounded freeze-dried leaves was approximately 0.24 µg/g, this is similar to what was seen in the mock levels of JA at dawn (0.3-1.6 µg/g, average = 0.7 µg/g) and night (0.2 – 5.6 µg/g, average = 1.43 µg/g). Absolute JA levels in mechanically wounded leaves was approximately 20,000 pg/10mg of tissue (Forcat *et al.,* 2008), this is similar to the JA levels in inoculated tissue harvested between 16-18 hpi and 20 hpi tissue after night inoculations (Fig.3.16).

Previous research has show that JA levels are rhythmic throughout the 24 h day (Goodspeed *et al.* 2012). After entrainment under 12:12 LD conditions, then

constant DD, JA peaks in the middle of the day (at approximately ZT 6) (Goodspeed *et al.* 2012). To further validate data in this study JA levels of mock samples were plotted against time of day to see if the same circadian profile was exhibited (Fig.3.15).

As outlined in methods, plants to be inoculated at dawn or night were kept in separate out of phase cabinets so the same fungal inoculums could be used. Plants were mock inoculated at dawn (ZT 0) and leaves were harvested 12 – 20 hpi (corresponding to ZT 12 – 20 (grey line in Fig.3.15)). Plants were mock inoculated at night (ZT 18) and leaves were harvested 12 – 20 hpi (corresponding to ZT 6 – 14 (black line in Fig.3.15)). ZT 12 and ZT 14 in Fig.3.15 have two data points collected from plants in two different cabinets. Although the cabinets are different, the conditions were identical and as such it was expected the JA levels would be similar in the mock samples collected at these time points. At ZT 12 plants from both cabinets exhibited very similar levels of JA, however, at ZT 14 plants from both cabinets show a large difference in JA levels (approx. difference between the two data points = 1400 pg/10mg of tissue) (Fig.3.15).

It would be expected that the JA levels would peak around ZT 6 in mock samples based on research by Goodspeed *et al.,* (2012) and that the overlapping time points from both cabinets would give similar levels of JA. However, the data in Fig.3.15 displayed two peaks in JA levels at ZT 8 and ZT 14, although neither of these points correspond with the data in Goodspeed *et al*., (2012) a peak at ZT 8 could be comparable given experimental differences.

The lack of circadian synthesis of JA could be due to the experimental protocol. The leaves are detached from the full plant and placed onto a phyto agar tray, then sampled every 2 hours by flash freezing. Both black and grey lines in the mock treatment samples (Fig 3.15) show a peak in JA 4 hours after detachment. It is therefore likely the plant wound response is playing a major role in inducing this JA peak given the similarities between the two peaks following inoculation (indicated in fig 3.15).

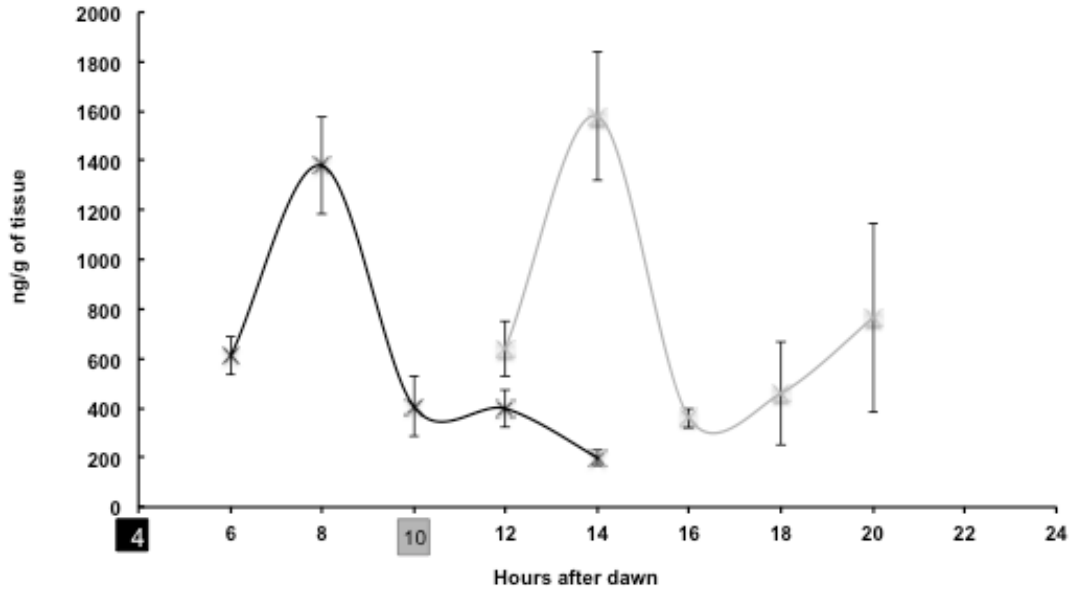


Figure 3.15 – Jasmonic acid levels in mock samples. Four week-old Col-0 leaves were detached and inoculated with mock suspension at dawn (ZT 0) or night (ZT 18) under LL conditions and samples were harvested every 2 hours between 12-20 hpi (dawn inoculation – hpi corresponds to ZT 12 – 20 (grey line) night inoculation hpi corresponds to ZT 6 – 14 (black line)). Detachment times are highlighted on the x-axis. Hormone JA measurements were then carried out by ultra-performance liquid chromatography mass spectrometry (UPLC-MS) (as outlined in Forcat *et al.,* 2008). Data shown are mean values of 3 biological replicates ± SEM.

The differences between the data in Fig 3.15 and Goodspeed *et al.,* (2012) could be due to different experimental set-ups. Goodspeed *et al.,* (2012) entrained plants under 12:12 LD conditions then placed them in DD for 24 h prior to data collection, whereas the data in Fig.3.15 was collected after plants were entrained under 16:8 LD conditions followed by 24 h in LL then mock inoculated and data collected from 12 – 20 h later. Differences in day length as well as light level differences during leaf harvesting could lead to differences in JA levels. JA levels have previously been shown to be dependent upon light wavelength (Cerrundo *et al.,* 2012).

Alternatively the difference between the two datasets could be due to the measurement protocols employed. Goodspeed *et al.,* (2012) measured jasmonates (including 12-oxo-phytodienoic acid (OPDA), MeJA and JA) using GC-MS, whereas the data in this study relied entirely upon measuring only JA levels using UPLC-MS. By measuring several forms of jasmonic acid Goodspeed

et al., (2012) ensured higher accuracy in their data. Measuring one component of JA (as in Fig.3.15) may not reflect JA activity, especially as JA-Ile is the active form of the molecule.

Measurements made in this study were preliminary and based on the above validation unlikely to be accurate. Never the less, JA levels were measured in mock and inoculated leaves, inoculated at either CT 24 or CT 42 and harvested every 2 h between 12 – 20 hpi (Fig.3.16). Again under LL conditions.

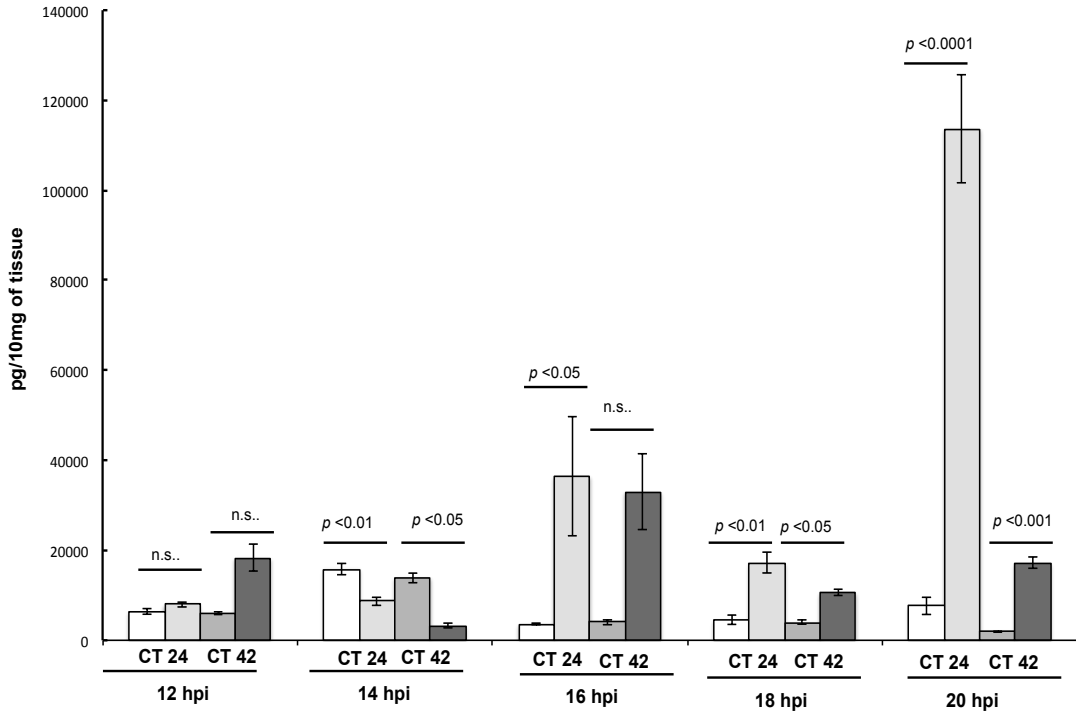


Figure 3.16 – Jasmonic acid levels are upregulated with more amplitude when plants are inoculated with *B. cinerea* at dawn compared to night. Four week-old Col-0 leaves were detached and inoculated with mock or *B. cinerea* suspension at dawn (CT 24) or night (CT 42) under LL conditions and samples were harvested every 2 hours between 12-20 hpi. Hormone JA measurements were then carried out by ultra-performance liquid chromatography mass spectrometry (UPLC-MS) (as outlined in Forcat *et al.,* 2008). Clear bars – dawn mock. Light grey – dawn infected. Medium grey – night mock. Dark grey – night infected. Student's T-Test was used to test whether hormone was significantly different between leaves inoculated with *B. cinerea* and mock. *p*-values for each comparison are shown. Data shown are mean values of 3 biological replicates ± SEM.

The general trend showed JA levels increased in infected compared to mock samples, as was anticipated (Fig.3.16). The exception to this was measurements

at 14 hpi at dawn or night (ZT 14 in the dawn sample and ZT 8 in the night sample) which both show JA levels to be significantly higher in the mock samples compared to the inoculated samples (Fig.3.16). This seems unlikely to be correct given the rest of the data and the well-documented role of JA in host *B. cinerea* defence.

Generally JA levels increased between 12–20 hpi in infected tissue, as was anticipated. 16 hpi is when the *B. cinerea* appears to initially elicit an increase in host JA levels (Fig.3.16). This increase is then dampened in samples harvested at 18 hpi and by 20 hpi *B. cinerea* appears to have elicited a significant response in samples, especially those inoculated at dawn (Fig.3.16).

JA upregulation in response to infection occurs at approximately 16 hpi (Fig.3.16). This timing of JA up-regulation between mock and inoculated samples partially corresponds with previous temporal transcriptional profiling carried out on *B. cinerea* infected leaf tissue (Windram *et al.,* 2012). In data by Windram *et al.,* (2012) numerous JA-biosynthetic genes in infected samples were expressed 12-14 hpi, this was followed by JA responsive genes being overrepresented in genes upregulated at 16 hpi in response to infection. This peak in JA responsive genes correlates to the measurement of JA levels in Fig.3.16. JA upregulation at 16 hpi also correlates with the previously outlined timing of *B. cinerea* growth. Between 16-20 hpi *B. cinerea* enters a rapid growth phase (Windram *et al.,* 2012) likely indicating colonisation of the plant. An increase in JA levels at 16 hpi is likely the plants response to this.

Interestingly, at 18 hpi a slight increase in JA levels in tissue infected at dawn compared to tissue infected at night is observed (Fig.3.16). This is the first indication the JA levels may be higher in response to inoculations that occur at dawn compared to those than are inoculated at night with relation to actual JA levels rather than JA transcriptional responses. Transcriptome profiling showed that genes related to JA signalling were up regulated more at 18 hpi in plants infected at dawn compared to plants infected at night. Therefore it would be

expected actual JA levels would increase prior to 18 hpi to allow for this downstream transcriptional signalling response.

The most striking observation here is at 20 hpi, where the level of JA in response to inoculation at dawn compared to inoculation at night is statistically different (Fig.3.16) and if these data are correct the differences observed here could play a major role in the time of day dependent immunity observed against *B. cinerea*. However, again this peak in JA is after hat would be expected based on the transcriptomic profiling results. Transcriptomic profiling revealed JA signalling responses were enhanced in response to infection more after dawn compared to night inoculations. These responses occurred at 18 hpi and by 22 hpi differential expression of these responses was not significantly different between the two inoculation times. It would therefore be expected the time-of-day dependent differences in JA levels between would have occurred prior to 18 hpi.

The timing differences seen between transcriptomic profiling and JA levels could be attributed to differences in infection progression. The inoculums, inoculation and infection incubation conditions were identical between the experiments for transcriptomic profiling and phytohormone measurements. One notable difference was the leaf ages employed in each experiment. Leaves five and six were used for phytohormone measurements whereas leaf seven was used for transcriptomic profiling. It could be this leaf age difference influenced the circadian regulated plant responses to *B. cinerea*. Indeed leaves of different ages on the same plants have shown varied circadian rhythms (Kim *et al.,* 2016).

This data was based on one biological replicate and is therefore likely the preliminary data and repeats are required to confirm the above.

3.3 Discussion

In Chapter 3 it was found that the time-of-day dependent differences observed in host response to *B. cinerea* are driven by the circadian clock differentially activating plant defences depending on the time of day of inoculation. When inoculated at subjective dawn (ZT 0) plants are much more resistant to *B. cinerea* compared to when inoculated at subjective night (ZT 18). Transcriptomic analysis of plants inoculated at subjective dawn or the subjective night revealed transcriptional defences appear amplified in response to inoculations that occur in at subjective dawn. For example, key TFs involved in *B. cinerea* defence (e.g. *MYB108* and *ERF6*) were found to be more upregulated or be expressed at a greater level in response to inoculations that occur at subjective dawn compared to a subjective night.

3.3.1 The role of light in *B. cinerea* infections

Indeed, it could be thought that the differences we have seen in the lesion measurements and fungal levels are due to something other than the circadian regulation of the transcriptome. Light plays a huge role in *B. cinerea* virulence and development (Canessa *et al*. 2013), JA regulation (Kazan and Manners, 2011) and plant immunity (Demkura & Ballaré, 2012) so it could be assumed this is the driving factor between the differences observed. However, Ingle *et al.,* (2015) observed differences in lesion sizes between plants in the same light phase (for example, plants in the dark phase displayed significantly different lesion sizes however lesions that occurred due to inoculations in the light phase were not significantly different from those that occurred in the dark phase . Further to this, the differences seen in lesion sizes that occurred at subjective dawn compared to subjective night persisted under constant light in different Arabidopsis ecotypes and this effect was completely abolished in dysfunctional clock mutants.

3.3.2 *B. cinerea* clock and Arabidopsis clock

The fungal circadian clock has been proposed to play a role in the time-of-day dependent variation observed in the outcome of inoculations (Hevia *et al.* 2015). Although it cannot be disputed the circadian clock in *B. cinerea* B05.10 strain employed by Hevia *et al.,* (2015) plays a role in the outcome of infections, the data in this chapter has proven the plant circadian clock is dominant when determining the outcome of infections with *B. cinerea* strain pepper. This chapter used the *B. cinerea* pepper isolate, which has clear development differences to the B05.10 isolate (Hevia *et al.,* 2015).

It was previously shown that *B. cinerea* strains are highly diverse in their responses to light (Canessa *et al.,* 2013). Approximately half of *B. cinerea* strains screened are light responsive (they produce different development structures depending on whether it is light or dark) or non-light responsive (they always form the same structures independent of the external light state) (Canessa *et al.,* 2013). The B05.10 strain is light responsive and produces sclerotia in the dark whereas the pepper isolate employed in this chapter always produces mycelia independent of the light source.

Moreover, the *B. cinerea* pepper strain employed in this work received no light entrainment prior to inoculation. It is kept under constant dark conditions up until conidiospores are detached for infections, and then all experiments were carried out under constant light and temperature conditions (unless otherwise stated). The BO5.10 strain was also employed in this chapter; it was cultured under the same DD and constant temperature conditions as the pepper strain. After this culturing the BO5.10 strain used to inoculate plants on the at subjective dawn or subjective night under LD or LL conditions the lesion sizes showed the same profile as the pepper strain, when plants were inoculated at subjective dawn with either strain they showed smaller lesion sizes compared to when inoculated at subjective night.

This absence of circadian entrainment in the culturing of both Botrytis strains means neither strain can have developed a circadian rhythm. However, the plants used in these studies were entrained under LD cycles for 4 weeks prior to inoculations hence the plants were the only organisms with running circadian clocks, therefore the only organisms that could be responsible for the time of day dependent differences in defence that have been observed in this chapter.

Hevia *et al.,* (2015) demonstrated Col-0 plants entrained for 4 weeks under 12 h LD conditions and then inoculated at subjective dawn or night with B05.10 (also entrained for 14 days under 12 h LD conditions) under LD or LL or DD conditions exhibited smaller lesion sizes when inoculated at subjective dawn compared to a subjective night (Hevia *et al.,* 2015). Although day lengths and fungal culturing conditions differ, the LD and LL results obtained by Hevia *et al.,* (2015) are in agreement with the results presented in this chapter. One potential disagreement with the results in this chapter lies in the results Hevia *et al.,* (2015) obtained when screening a circadian clock mutant.

Hevia *et al.,* (2015) carried out the same experiment as above on an Arabidopsis circadian clock mutant, *CCA1-OX*, again under LD, LL and DD conditions after both fungal and plant entrainment. A *prr5/7/9* was also screened for circadian immunity, but under DD conditions only. The *CCA1-OX* maintained greater resistance to *B. cinerea* B05.10 when inoculated at subjective dawn compared to a subjective evening under DD and LD conditions (Hevia *et al.,* 2015). The *prr5/7/9* mutant also maintained a higher level of resistance when inoculated at dawn compared to night. Hevia *et al.,* (2015) concluded this phenomenon was due to the fungal clock dominating the infection outcome. However, when inoculated at dawn and night under LL conditions the *CCA1-OX* mutant lost this rhythmicity in defence against *B. cinerea* B05.10 (Hevia *et al.,* 2015). Under LL conditions the mutated clock plant mutant lost any differences between lesion sizes formed from dawn or night inoculations. How could the plant clock mutant lose the time-of-day dependent immunity under only one of the light conditions?

The *CCA1-OX* mutant is not completely arrhythmic under all conditions (Green *et al.,* 2002). Although *CCA1-OX* plants lack the ability to anticipate dawn, these mutants still exhibit robust diurnal oscillations in *CCA1/LHY* gene expression when grown under LD conditions (Green *et al.,* 2002). Hence *CCA1-OX* exhibited wild-type time-of-day dependent susceptibility under LD conditions as the clock was still running and genes were still being rhythmically expressed in the plant. The fungal clock could have contributed to this time-of-day dependent susceptibility profile but the plant clock will have also had a strong role. It would be interesting to see the outcome of identical LD experiments using the same entrained B05.10 strain with a truly arrhythmic plant mutant such as *prr5/6/9*, which Hevia *et al.,* (2015) later employed for a DD screen.

One circadian clock mutant (*elf3*) has defective circadian rhythms in LL but not DD (Hicks *et al.,* 1996). The *CCA1-OX* and *prr5/7/9* mutants have shown no rhythmicity under LL or DD conditions (Nakamichi *et al.,* (2005); Nakamichi *et al.,* (2009); Wang and Tobin, (1998)). However, the observation that *prr5/7/9* mutant displays no rhythmicity under DD is based entirely on CCA1 bioluminescence patterns 12 h after incubation under DD conditions (Nakamichi *et al.,* 2009). Prior to 12 h CCA1 may show rhythmicity in its expression due to the prior entrainment however this needs further investigation. Moreover, the *CCA1-OX* showed peaks in the expression levels of several genes (CCA1 and Lhcb1) albeit at earlier time points and within the first 12 h of being in DD conditions (Wang and Tobin, 1998). Further experiments under DD conditions should account for these residual rhythms and keep plants under DD for at least 12 h prior to any experimental treatments. Both clock mutants when inoculated at subjective dawn and night were not kept in DD prior to inoculations hence this may have impacted the outcome of the interaction. Rhythmic genes within the first 12 h of the inoculation period may have still been under circadian regulation; hence the clock may have affected the infection outcome.

Another possibility is that under DD conditions the fungal clock plays a dominant role in governing the infection outcome and that the plant clock plays a dominant role under LD and LL conditions.

The loss of circadian immunity under LL conditions is unsurprising as the *CCA1-OX* mutation renders the plant completely arrhythmic under LL conditions (Daniel *et al.,* (2004); Green *et al.,* (2002); Wang and Tobin (1998)). Therefore, genes are not cycling and any role the plant clock plays in the defence response will be abolished. Again, it would be interesting to see the outcome of identical LL experiments using the same entrained B05.10 strain with a *prr5/6/9* mutant.

Ingle *et al.,* (2015) inoculated two circadian clock mutants (*cca1 lhy* and *elf3-1*) at subjective dawn or subjective night under LL conditions after four weeks of LD entrainment. It was observed both mutants completely lost any differences between lesion sizes from inoculations occurring at the two time points.

It is likely both the fungal and plant circadian clock contribute the differences in immunity under natural LD conditions. However, the data shown in this chapter shows the plant circadian clock dominates the observed time-of-day dependent differences in susceptibility to *B. cinerea* under LD and LL conditions. Neither of the fungal strains employed in this work received any circadian entrainment and inoculations at subjective dawn gave rise to smaller lesions than those subjective night inoculations. Furthermore, two dysfunctional plant clock mutants that are arrhythmic under LL conditions lost the observed differences in immunity to *B. cinerea* under LL conditions.

A future experiment to further eliminate any doubt that the circadian clock within the plant is the driving force behind this rhythmic interaction would be to use an arrhythmic *B. cinerea* strain under the infection conditions used throughout this chapter. If the plant still shows a difference in dawn compared to night resistance levels when inoculated with an arrhythmic *B. cinerea Δbcfrq1* mutant line and/or the *BcFRQ1* over-expression line (both employed in

Hevia *et al.,* 2015) then this would fully confirm the plant clock is regulating this interaction.

3.3.3 Comparison of rhythmic defence against biotrophic and necrotrophic pathogens

As previously mentioned, the circadian clock has been shown to play a crucial part in time-of-day dependent defences against the bacterial pathogen, *P. syringae* (Bhardwaj *et al.,* 2011) and the oomycete pathogen, *Hpa* (Wang *et al.,* 2011). Similar to results presented in this chapter, plants display maximal resistance to *P. syringae* when inoculated at dawn and increased susceptibility when inoculated in an evening (Bhardwaj *et al.* 2011). However, given the antagonism that is thought to exist between necrotrophic and biotrophic defence pathways in plants (Koornneef & Pieterse, 2008) it might be expected that both defences are 'switched-on' at different times of day and hence peaks in resistance to both necrotrophs and biotrophs would not occur at the same time.

However, both peaks in resistance are unlikely to occur at the same time in the plant, *P. syringae* quickly attacks the plant and causes transcriptional changes in the host within 2 hours (Truman *et al.,* 2006), whereas *B. cinerea* does not initiate any host transcriptional responses till 10 hpi (Windram *et al.,* 2012). This could be due to *B. cinerea* spores not staying largely intact and till 10 hpi and then appearing to germinate between 10 – 12 hpi (Fig.3.4).

Hence, the plant is sensing and reacting to the pathogens at two different time points. If inoculated at CT 24 (subjective dawn) with *P. syringae* or *B. cinerea*, the host would sense and start responding to the former by CT 26 (morning) and the latter, *B. cinerea* at CT 34 (late afternoon). Therefore, in the plant, the defences to each pathogen are not peaking at the same time; the defences against *P. syringae* will be most active and responsive on a morning in the plant. Whereas the defences in the plant against *B. cinerea* will not be the most

responsive on at dawn, they will be most responsive between 10-12 h after dawn, when the fungus is actually perceived by the plant.

JA and SA are the major hormones involved in defence against necrotrophs and biotrophs respectively (as reviewed in Glazebrook, 2005). It has been shown under non-stress conditions JA peaks in the middle of the day whereas as SA peaks in the middle of the night (under 12:12 DD conditions) (Goodspeed *et al.,* 2012). This peak in JA levels corresponds with the observed maximal resistance to *B. cinerea* when plants are inoculated at subjective dawn. If a plant is inoculated at ZT 0 and begins influencing the plant transcriptome 10 hpi then the plant perceives the fungus at ZT 10, this approximately correlates to when the JA levels have been seen to be high (Goodspeed *et al.,* 2012). This also correlates to a peak in JA related gene expression observed at ZT 10 under mock conditions in Windram *et al,* (2012).

3.3.4 The circadian clock is differentially activating the defence pathway in response to dawn compared to night inoculations

The link between the circadian clock and defence against *B. cinerea* does not lie in the role the circadian clock plays in regulating stomatal aperture at different times of day (Dodd *et al.,* 2005), as this pathogen does not appear to enter the host via stomatal penetration (Fig.3.3).

Furthermore, this chapter has shown the fungal development varies depending on time of inoculation. At 12 hpi there are fewer hyphae when plants are inoculated at subjective dawn compared to the subjective night (Fig.3.4). Later time points then show a delayed development of 'claw-like' infection structures in plants inoculated at dawn compared to subjective night (Fig.3.6). This is possibly due to the plant defences in response to dawn inoculations having an influence on the initial hyphal numbers. It could be that the plant is somehow slowing the fungal development steps; future work would need to be done to confirm this.

Future work could include carrying out gene expression studies of *B. cinerea* during the infection process. The fungus develops throughout the infection process, for example the fungus forms appressorium to enter the plant, gene expression changes then correlate with this development. For example appressorium-mediated penetration of host tissue requires the *BcPLS1* gene that encodes a membrane protein specific to the appressorium (Gourgues *et al.,* 2004). Genes specific to each fungal development stage during the infection stage could be categorised using series transcriptome data coupled with visual microscopy techniques. Once each infection stage (germination, appressorium formation, claw formation etc.) had several genes specific to that stage identified, these genes could then be used to track fungal development in detail. Expression studies of these fungal genes in plants inoculated at dawn or night would give an accurate quantifiable method for identifying which fungal stage is slowed in inoculations that occur at dawn compared to night.

3.3.4.1 The circadian clock is gating the JA signalling pathway

This chapter uncovered the differential regulation of the JA-signalling pathway in response to inoculations that occur at subjective dawn compared to those at subjective night (Fig.3.8). JA responsive signalling genes appeared to be more responsive to *B. cinerea* inoculations that occurred at dawn compared to those that occurred at night. This follows the hypothesis formed by Shin *et al.,* (2012) that the circadian clock is gating the responses of the JA stress-signalling pathway.

The biosynthesis of JA is under circadian regulation and levels of the phytohormone display rhythmicity in unstressed tissue (Goodspeed *et al.,* 2012). TOC1 could be largely responsible for the rhythmicity of JA biosynthesis as it directly binds to three genes *LIPOXYGENASE (LOX)* genes that encode 13-LOX enzymes in involved in the very first initiation steps JA biosynthesis. Genes involved in signalling and biosynthesis of JA are directly bound by circadian clock components (Appendix 1) and both signalling and JA biosynthesis genes maintain low basal levels in unstressed tissue (Shin *et al.,* 2012). JA levels are

also low in unstressed tissue (Fig.3.15). However, in infected tissue the level of JA signalling and biosynthesis related gene expression greatly increases in response to *B. cinerea*. It could therefore be the case that given the differences in both tissues, the regulation of JA biosynthesis and signalling in unstressed tissue and infected tissue are controlled by separate circadian clock components. The regulation in unstressed tissue is likely separate to the gating response observed in the JA related stress response pathway (Shin *et al*., 2012).

Why the clock would be gating this specific JA pathway in such a regulated and complex manner is yet to be elucidated. The circadian clock and phytohormones both play major roles in fine-tuning the internal biology of an organism to the external environment so cross talk between them is vital. It is likely that the JA signalling stress response is highly costly in terms of energy expenditure and therefore having high levels at a particular time of day without any stress stimuli is wasteful. Consequently having a gating mechanism in place allowing JA stress signalling to respond more rapidly and/or with greater amplitude in response to *B. cinerea* attack when it is most likely will give plants an evolutionary advantage.

3.3.5 Potential regulatory links between the circadian clock and host defences against *B. cinerea*

Analysis of transcriptomic profiling within this chapter revealed several likely candidates linking the circadian clock to the defence response. Future work should focus on screening mutants of these genes for differences in resistance to *B. cinerea* following dawn or night inoculations. An example candidate is *MYB108*. *MYB108* gene expression increased more between mock and inoculated samples when inoculated dawn compared to night (Fig.3.13).

MYB108 (also known as BOS1 *(BOTRYTIS-SUSCEPTIBLE 1)*) is implicated in host defence against *B. cinerea*. *MYB108* gene expression greatly upregulated in response to *B. cinerea* inoculation (Mengiste *et al.,* (2003); Windram *et al.,*

(2012)) and mutants with reduced *MYB108* gene expression exhibit significantly increased susceptibility to *B. cinerea* (Mengiste *et al.,* 2003).

The induction of *MYB108* in response to *B. cinerea* is dependent on the JA receptor, COI1. A *coi1* mutant displayed both delayed and reduced *MYB108* induction in response to *B. cinerea* (Mengiste *et al.,* 2003). This *COI1* dependent expression suggests the JA pathway regulates *MYB108* expression.

The circadian clock (specifically *TIC*) gates the expression levels of *COI1* in response to JA application (Shin *et al.,* 2012). Given that *MYB108* is regulated by *COI1*, this could be the way in which the clock is regulating *MYB108* transcript levels, and so the host defence network against *B. cinerea,* differently in response to inoculations at dawn or night. To confirm this hypothesis *MYB108, COI1* and *TIC* mutants could be screened for differences in resistance to *B. cinerea* when inoculated at dawn or night.

Chapter 4: *JAZ6* links the circadian clock and plant defence against *Botrytis cinerea*

Some results from this chapter are published in Ingle *et al.,* (2015)

4.1 Introduction

Evidence in the previous chapter lead to the hypothesis that increased resistance against *Botrytis cinerea* after inoculation at subjective dawn compared to subjective night was due to the differential regulation of the JA signaling pathway. From this hypothesis it was then asked, how is the JA signaling pathway influencing the circadian regulation of defence? To answer this question, JA signalling mutants were screened for *B. cinerea* resistance at subjective dawn compared to subjective night.

It was decided to focus on JAZ proteins as they regulate a number of TFs involved in JA signalling cascades through protein binding. These TFs include MYC2 (Chini *et al.* (2007); Thines *et al.* (2007)) a master regulator of many JA responses including anthocyanin biosynthesis, root growth, wounding responses and host defence against necrotrophic fungi (Dombrecht *et al.* (2007); Lorenzo *et al*. (2004)). Other TFs bound by the JAZ proteins are EIN3 and EIL1; both are central activators of the ET response and this binding is postulated to contribute the cross talk between the JA and ET pathways (Zhu *et al.,* 2011). Moreover, the promoter regions of four *JAZs* (*1/5/6/9*) were directly bound by at least one central oscillator protein (see Chapter 1) showing these proteins are regulated by the circadian clock and then go on to regulate the JA signalling pathway. Although the interactions between specific TFs or families of TFs have been investigated for interactions with JAZ proteins, no TF wide study has investigated JAZ binding.

The JA signalling pathway and the role JAZ proteins in this pathway has been extensively reviewed (see Chapter 1). In brief, the JAZ proteins mediate signalling events triggered by JA; in times of low JA JAZ proteins repress key TFs

in JA signalling cascades (Fernández-Calvo *et al.* (2011); Thines *et al.,* (2007)). Increased JA levels lead to the binding of JA-Ile (the bioactive form of JA) to the JA receptor, COI1. COI1 is a component of an ubiquitin E3 ligase complex which upon the perception of JA-Ile ubiquitinates JAZ proteins, leading to their degradation by the 26S proteasome (Chini *et al.* (2007); Thines *et al.* (2007)). Once JAZ proteins are destroyed, many TFs (such as MYC2/3/4) are free from JAZ-mediated repression and can activate their downstream targets (Chini *et al.* (2007); Fernández-Calvo *et al.* (2011); Thines *et al.,* (2007)).

The JAZ proteins are a family of at least 12 transcriptional repressors (Chung *et al.,* 2010), which show a high level of functional redundancy. Single T-DNA insertion lines corresponding to single knockouts in JAZ2/5/7/9 loci displayed no JA related phenotype (Thines *et al.,* 2007). Moreover, two single T-DNA insertion lines corresponding to single knockouts in the JAZ5 and JAZ10 loci displayed wild-type phenotypes against *Pseudomonas syringae* (de Torres Zabala *et al.*, 2015). Whereas a double JAZ5 and JAZ10 knockout line displayed enhanced susceptibility to *P. syringae* (de Torres Zabala *et al.*, 2015). Most of the JAZ proteins have also been shown to be able to form both hetero- and homodimers with other family members via the ZIM domain (Chini *et al.,* 2009). The exact function of this dimerization *in-planta* is as yet unknown. Given this dimerization ability and functional redundancy within the JAZ family, triple mutants were first employed in this chapter.

JAZ proteins were shown to play a key role in mediating the time-of-day dependent resistance to *B. cinerea*. To gain insight into how proteins could be doing this attention was focused to TFs as binding targets of the JAZ proteins are often TFs (for example, MYC2/3/4 (Fernández-Calvo *et al.* 2011), EIN3 and EIL1 (Zhu *et al.,* 2011) PAP1 and GL1 (Qi *et al.,* 2011)). It was therefore asked, what TFs are JAZ proteins binding to which regulate this time-of-day dependent difference? To answer these questions an extensive yeast-two-hybrid (Y2H) screen was performed against the majority of TFs uncovered in Arabidopsis (screened 1956 of 2400) (Pruneda-Paz *et al.,* 2014). Y2H detects protein-protein interactions by employing the *Saccharomyces cerevisiae GAL4* as a

reporter gene activation system. The GAL4 TF is split into two domains (the activation domain (AD) and the binding domain (BD)), each domain fused to a separate protein and it is only when the two proteins physically interact that the GAL4 TF can activate expression of the reporter gene (Fields and Song, 1989). This system only gives an indication of what proteins are able to bind and not what proteins actually bind *in-planta*. Further *in-planta* techniques such as Bimolecular fluorescence complementation (BIFC) and Co-Immunoprecipitation (Co-IP) should next be performed to validate the interaction observed in this Y2H screen.

4.2 Results

4.2.1 Specific *JAZ* mutations alter the time-of-day difference in resistance to *B. cinerea*

4.2.1.1 A triple *JAZ* mutant exhibits altered time-of-day differences in resistance to *B. cinerea*

To address possible functional redundancies between members of the JAZ family, triple JAZ mutants were obtained (from Prof. Murray Grant, School of Life Sciences, University of Warwick). Triple mutants containing T-DNA insertions in the *JAZ5, JAZ6, JAZ7* or *JAZ10* loci were selected for.

JAZ5 and *JAZ10* were selected as together they have been shown to positively regulate host defence against *P. syringae* (de Torres Zabala *et al.*, 2015). Furthermore, *JAZ10* has also been shown to be a key link between plant light responses and host defence against *B. cinerea* (Cerrundo *et al.,* 2012; Leone *et al.,* 2014). *JAZ6* was included in this screen, as it has been shown to be involved in the suppression of the SA pathway to increase resistance to *P. syringae* in tomato (Ishiga *et al.*, 2013). *JAZ5* and *JAZ6* are also targets of the *P. syringae* effector *HopZ1a* (Jiang *et al.,* 2013); generally effector targets are essential for the defence response. For example, *P. syringae* avirulence proteins (AvrPto) inhibit flagellin induced plant immune responses by inhibiting receptor like kinases (RLKs) such as Flagellin Sensing 2 (FLS2) (Zong *et al.,* 2008). *P. syringae* lacking AvrPto show reduced virulence in Arabidopsis (Zong *et al.,* 2008). Finally, *JAZ7* was included in this screen due to its key role in fungal defence pathways, as demonstrated by the *jaz7-1D* overexpression line showing increased susceptibility against *Fusarium oxysporum* (Thatcher *et al.,* 2016).

Col-0 and two triple mutants (*jaz5/6/10* and *jaz6/7/10*) plants were entrained for 4 weeks under 16 h light dark (LD) conditions and then inoculated at subjective dawn or subjective night under LD or constant light (LL) conditions as described in Chapter 2. Plants inoculated at subjective dawn (CT 24) or subjective night (CT 42) in LL were transferred to LL conditions 24 h prior to

inoculation and the remainder of the infection was left to progress under LL conditions. Lesions were measured 72 h after inoculation (Fig 4.1).

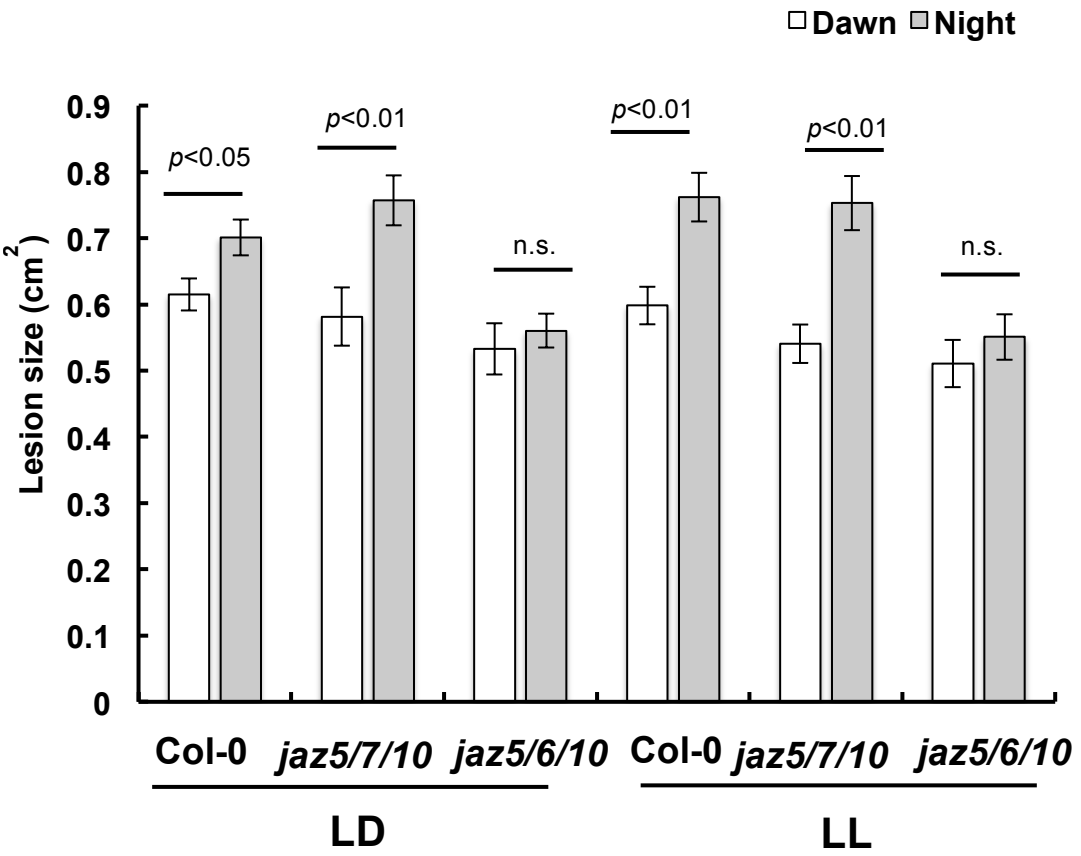


Figure 4.1 – *jaz5/6/10* shows no difference in resistance to *B. cinerea* inoculations at dawn compared to those at night. Leaves from four-week-old plants were inoculated with *B. cinerea* under LD conditions at dawn (ZT 0) or night (ZT 18) or under LL conditions at subjective dawn (CT 24) or night (CT 42) and lesion area measured at 72 hpi. Data shown are mean lesion sizes ± SEM of wild-type (Col-0) leaves versus *jaz* mutants (n ≥30 per line and inoculation time). Student's T-Test was used to test whether lesion size was significantly different between leaves inoculated at dawn versus night. *p*-values for each comparison are shown.

Col-0 leaves inoculated at dawn (ZT 0) (or subjective dawn (CT 24)) compared to night (ZT 18) (or subjective night (CT 42)) showed a significant difference in lesion sizes, with the smallest lesion sizes following dawn or subjective dawn inoculations (Fig.4.1).

Col-0 leaves maintained this difference under LD and LL conditions (Fig.4.1). Results for Col-0 and *JAZ* mutant lines between the independent LD and LL

experiments were very similar suggesting day length did not affect susceptibility. This similarity also showed that the circadian clock rather than light *per se* exhibits a greater influence on the plant immune response under conditions employed in this chapter (Fig.4.1).

jaz5/7/10 showed no difference in susceptibility levels to *B. cinerea* when compared to Col-0 under LD or LL conditions. *jaz5/7/10* maintained higher resistance levels in response to inoculations at dawn compared to night under LD and LL conditions (Fig.4.1). However, *jaz5/6/10* lost any time-of-day dependent differences in resistance levels to *B. cinerea*, night time susceptibility was lost in this line under LD and LL conditions (Fig.4.1).

The gene mutation differing between the *jaz5/7/10* line that did not alter the defence response from what was observed with Col-0 and *jaz5/6/10*, which lost time-of-day dependent resistance to *B. cinerea* unlike Col-0, is a knockout in the *JAZ6* gene. *JAZ6* may therefore be involved in the circadian clock's regulation of defence against *B. cinerea.* To identify if one singular *JAZ* in the *jaz5/6/10* mutant was indeed responsible for the loss of time-of-day dependent resistance to *B. cinerea*, single and double mutant combinations of *JAZ5, JAZ6* and *JAZ10* were screened exactly as outlined above for LD inoculations.

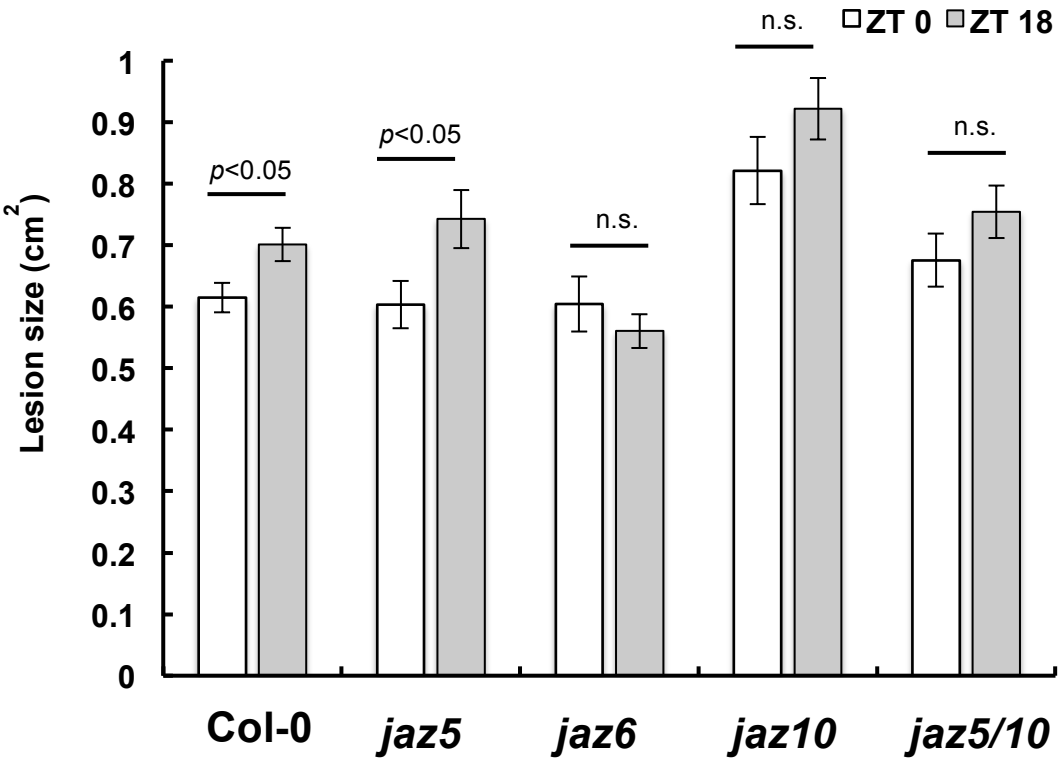


Figure 4.2 – *jaz6* shows no difference in resistance to *B. cinerea* inoculations at dawn compared to those at night under cyclic light conditions. Leaves from four-week-old plants were inoculated with *B. cinerea* under LD conditions at dawn (ZT 0) or night (ZT 18) and lesion area measured at 72 hpi. Data shown are mean lesion sizes ± SEM of wild-type (Col-0) leaves versus *jaz* mutants (n ≥30 per line and inoculation time). Student's T-Test was used to test whether lesion size was significantly different between leaves inoculated at dawn versus night. *p*-values for each comparison are shown.

As expected Col-0 showed significantly larger lesion sizes on leaves inoculated at night compared to those inoculated at dawn. A single *JAZ5* mutant (*jaz5*) showed the same phenotype as Col-0, with significantly larger lesions if inoculated at night compared to dawn (Fig.4.2). Both *jaz10* and a double mutant of both *JAZ5* and *JAZ10* (*jaz5/10*) displayed marginally larger lesion sizes when inoculated at night compared to at dawn (Fig.4.2). JAZ5 or JAZ10 could have a role in linking the plant circadian clock and defence response.

The single *jaz6* mutant showed no difference between lesions formed from inoculations at dawn or night (Fig.4.2). *JAZ6* could be linking the plant circadian clock to the defence pathway against *B. cinerea*.

4.2.1.2 *JAZ6* plays a key role in modulating time of day dependent differences in susceptibility to *B. cinerea*

To confirm the phenotype observed with the *jaz6* mutant was due to reduced *JAZ6* expression, Ingle *et al.,* (2015) performed qPCR analysis of *JAZ6* 18 h after inoculation or mock inoculation at dawn (ZT 0) (Fig.4.3). This confirmed the transcriptional induction of *JAZ6* in response to *B. cinerea* inoculation was reduced in the *jaz6* mutant compared to Col-0.

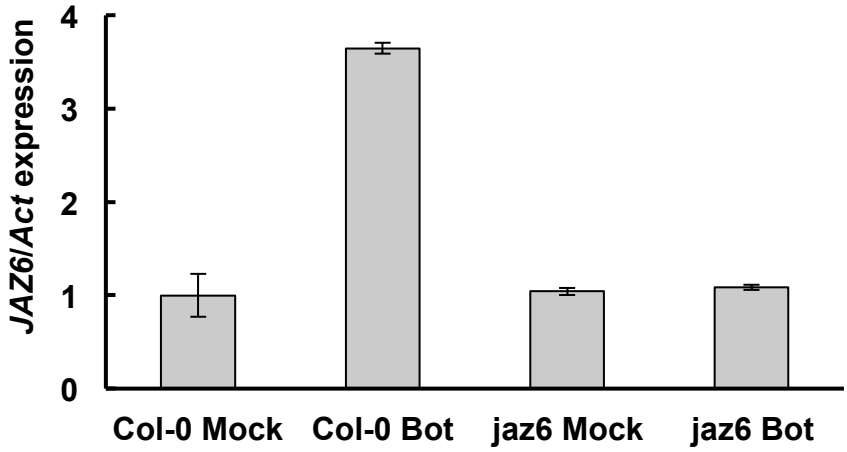


Figure 4.3 - Expression of *JAZ6* in the *jaz6* mutant line and Col-0 18 hpi with *B. cinerea* or mock control. Detached leaves from four week-old plants grown under LD were inoculated with *B. cinerea* spores (Bot) or half-strength grape juice (Mock) at dawn (ZT 0) and tissue harvested for RNA extraction 18 hpi. Values shown are mean expression values (normalised to *Actin2* expression) from three biological replicates ± SEM. (Reproduced with permissions from Ingle *et al.,* 2015)

After confirming *JAZ6* expression was reduced in the *jaz6* mutant, a qPCR of the *JAZ6-OX* line was carried out. This line shows constitutive over expression of *JAZ6* by placing the gene under a *35S* promoter (line generated by Prof. Ari Sadanandom group at Durham University). Plants were grown under LD conditions for four weeks, placed under LL for 24 h then inoculated at ZT 0 (subjective morning) with *B. cinerea* or mock inoculum. Leaves were harvested 18 hpi and *JAZ6* expression relative to *PUX1* was determined (Fig.4.4). *PUX1* was selected as it is constitutively expressed and does not alter in expression in response to *B. cinerea* infection (Windram *et al.,* 2012). This confirmed the

transcriptional induction of *JAZ6* in response to *B. cinerea* inoculation was increased in the *JAZ6-OX* mutant compared to Col-0.

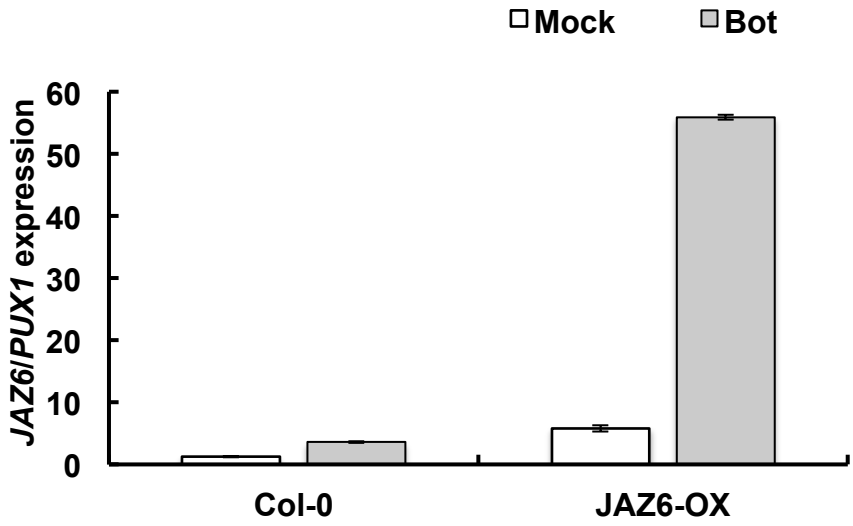


Figure 4.4 - Expression of *JAZ6* in the *JAZ6-OX* mutant line and Col-0 18 hpi with *B. cinerea* or mock control. Detached leaves from four week-old plants entrained under LD conditions were placed in LL then inoculated with *B. cinerea* spores (Bot) or half-strength grape juice (Mock) at dawn (ZT 0) and tissue harvested for 18 hpi. Values shown are mean expression values (normalised to *PUX1* expression) from two biological replicates ± SEM.

The qPCR of *JAZ6* in the *JAZ6-OX* line was carried out on only two leaves per time point for one *JAZ6-OX* line and Col-0 hence this data is preliminary and ideally would have been repeated to confirm the overexpression of *JAZ6* in this line. Each plant was first checked for the presence of the *JAZ6* overexpression construct (*35S:JAZ6:GFP*) using *35S* and *JAZ6* specific primers (Appendix 3). Once the construct was confirmed plants were then screened for expression levels of *JAZ6*.

The *JAZ6-OX* line showed a three-fold increase in *JAZ6* expression under mock conditions and 15-fold increase after inoculation conditions (Fig.4.4). In this line, the constitutive *35S* promoter regulates *JAZ6* expression and it is no longer under the control of its native promoter. Therefore *JAZ6* in this line would not be anticipated to change in response *B. cinerea* inoculation and levels of *JAZ6* in the *JAZ6-OX* line should remain consistent.

It is possible that the *35S* promoter is responsive to *B. cinerea* infection, hence the upregulation of *JAZ6* in the infected compared to mock overexpressor line. However, this scenario is unlikely, given several genes have been placed under the regulation of the *35S* promoter and no differential expression in response to infection to *B. cinerea* has been documented (Berrocal-Lobo *et al.* (2002); Lionetti *et al.* (2007); Zhao *et al.* (2012)). The second explanation is that the primers employed in this study are targeting both the endogenous *JAZ6* and the *JAZ6* under the control of the *35S* promoter. As such, it could be that the high levels of protein produced by the *35S:JAZ6* construct are creating a feedback loop and increasing the endogenous levels of *JAZ6* under its native promoter. There are two possible ways in which this could happen, either JAZ6 could auto regulate or increased JAZ6 leads to some indirect upregulation. A third explanation could be related to the posttranscriptional modification of JAZ6 mRNA. Previous research has shown SM-like (LSM) proteins 1-7 decap JAZ6 mRNA and the mRNA is subsequently degraded in the cytoplasm by enzymes such as EXORIBONUCLEASE4 (XRN4) making JAZ6 an unstable transcript (Golisz *et al.* (2013); Perea-Resa *et al.* (2012)). It is possible that this decapping takes place under mock but not infection conditions, hence why *JAZ6* is only marginally overexpressed under mock conditions even in the overexpressor line. However, given that the *JAZ6:OX* line was shown by PCR to contain the *35S:JAZ6:GFP* construct (Appendix 3) and the qPCR results, although not entirely reliable, show *JAZ6* is most likely over expressed in this line.

A further possibility is that the *JAZ6:OX* construct, expected to be expressed at greater levels under mock conditions, is silenced within the plant. This silencing is then antagonized by *B. cinerea*. It could be that whatever mechanism is silencing the construct is removed or interfered with by *B. cinerea*. This is possibly related the sRNAs secreted by *B. cinerea*, (Weiberg *et al.,* 2013). However, this requires further investigation.

The *jaz6* and *JAZ6-OX* mutant lines were confirmed to be repressed and overexpressed JAZ6 expression levels, respectively; the links *JAZ6* has with the clock and host *B. cinerea* defence were then investigated.

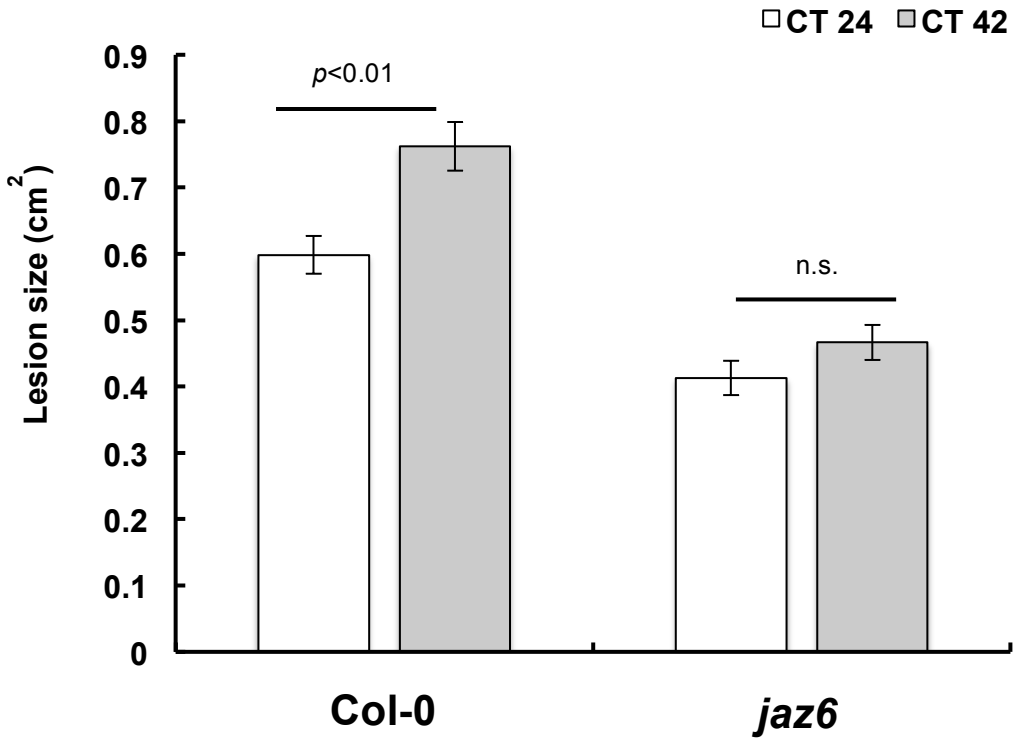


Figure 4.5 – *jaz6* shows no difference in resistance to *B. cinerea* inoculations at subjective dawn compared to those at subjective night under constant light conditions. Leaves from four-week-old plants were inoculated with *B. cinerea* under LL conditions at subjective dawn (CT24) or night (CT42) and lesion area measured at 72 hpi. Data shown are mean lesion sizes ± SEM of wild-type (Col-0) leaves versus *jaz6* mutant (n ≥60 per line and inoculation time). Student's T-Test was used to test whether lesion size was significantly different between leaves inoculated at dawn versus night. *p*-values for each comparison are shown.

Under LL conditions Col-0 maintained the dawn compared to night difference in resistance to *B. cinerea*, however *jaz6* repeatedly showed no difference between the two inoculation times (Fig.4.5). Given the above evidence it appears *JAZ6* is playing a vital role in circadian clock mediated resistance to *B. cinerea*. As a further means of confirmation a *JAZ6-OX* line was screened in the same way as outlined for previous experiments under LD conditions.

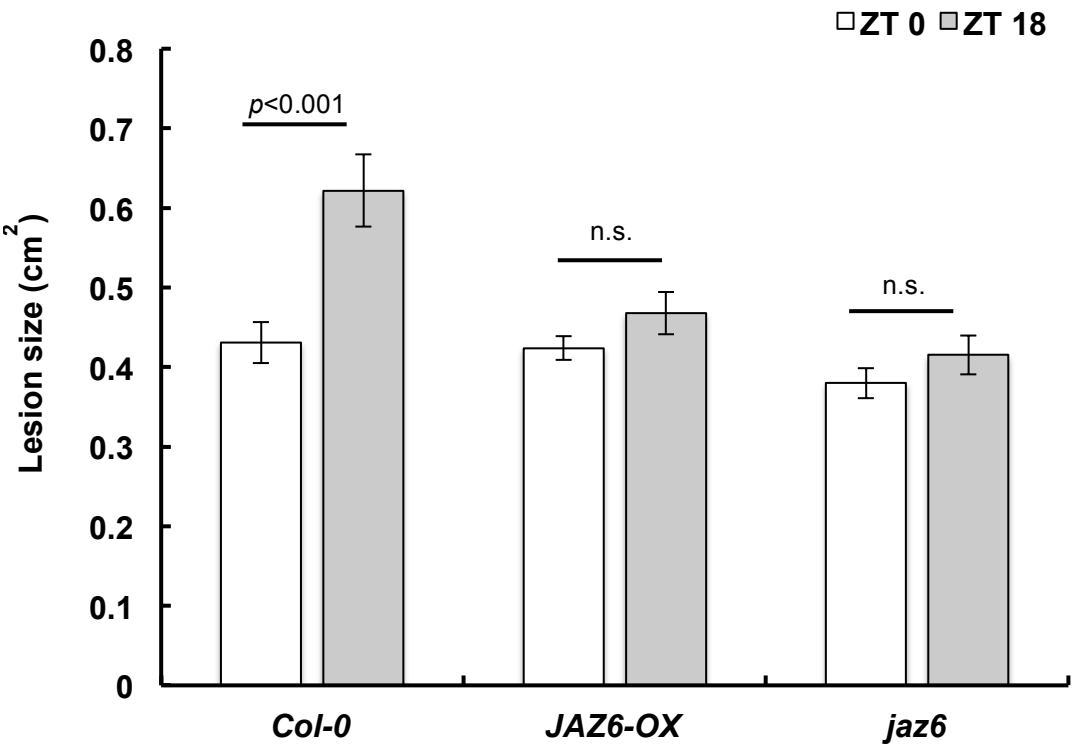


Figure 4.6 – *jaz6* and *JAZ6-OX* show no difference in resistance to *B. cinerea* inoculations at dawn compared to those at night under cyclic light conditions. Leaves from four-week-old plants were inoculated with *B. cinerea* under LD conditions at dawn (ZT0) or night (ZT18) and lesion area measured at 72 hpi. Data shown are mean lesion sizes ± SEM of wild-type (Col-0) leaves versus *jaz6* and *JAZ6-OX* mutants (n ≥30 per line and inoculation time). This is representative of two independent experiments. Student's T-Test was used to test whether lesion size was significantly different between leaves inoculated at dawn versus night. *p*-values for each comparison are shown.

Under cyclic LD conditions Col-0 once again showed a significant difference in lesion sizes formed from dawn compared to night inoculations, the *jaz6* mutant once again lost this difference. Furthermore, the *JAZ6-OX* line also lost this difference between lesion size formations when inoculated at dawn compared to night (Fig.4.6).

In summary, a *JAZ* triple mutant (*jaz5/6/10*), a *jaz6* and a *JAZ6-OX* mutant lost circadian driven resistance to *B. cinerea*. Furthermore, *JAZ6* has strong links to the circadian clock (Appendix 1), a rhythmic expression profile and has previously been shown to increase in expression in response to *B. cinerea* infection (Windram *et al.,* 2012). It appears JAZ6 is a key link between the

circadian clock and host *B. cinerea* defence. How *JAZ6* is linking the two pathways remains to be elucidated.

4.2.3 Protein-Protein interactions of JAZs

This section investigates how *JAZ6* is linking the circadian clock to the defence pathway against *B. cinerea* by investigating the potential protein-binding partners of JAZ6 *in-vitro* using Y2H. JAZ6 has previously been shown to mediate the JA signaling pathway by binding to, and repressing, TFs (Song *et al.,* 2013) so the protein was screened against the majority of known TFs in Arabidopsis (n = 1956) using Y2H (Appendix 5) (Pruneda-Paz *et al.,* 2014). This screen was carried out in a unidirectional manner (JAZ6:DB against TF library:AD). Mating plates were replica plated onto SD-Leu-Trp (SD-LT) (to check for successful diploid formation), SD-Leu-Trp-His (SD- LTH) and SD-Leu-Trp-Ade (SD-LTA) (growth on either SD-LTH or SD-LTA indicates successful protein-protein interactions).

4.2.3.1 The Y2H screen is reproducible

All interactions were tested three times (unless otherwise stated) using independent mating reactions which were then printed onto two selective media (Fig.4.7). As can be seen interactions were shown multiple times. Any results that only repeated once were dismissed as they were deemed to be false positives. As can be seen in Fig.4.7 the background growth in this screen was minimal with very few colonies forming.

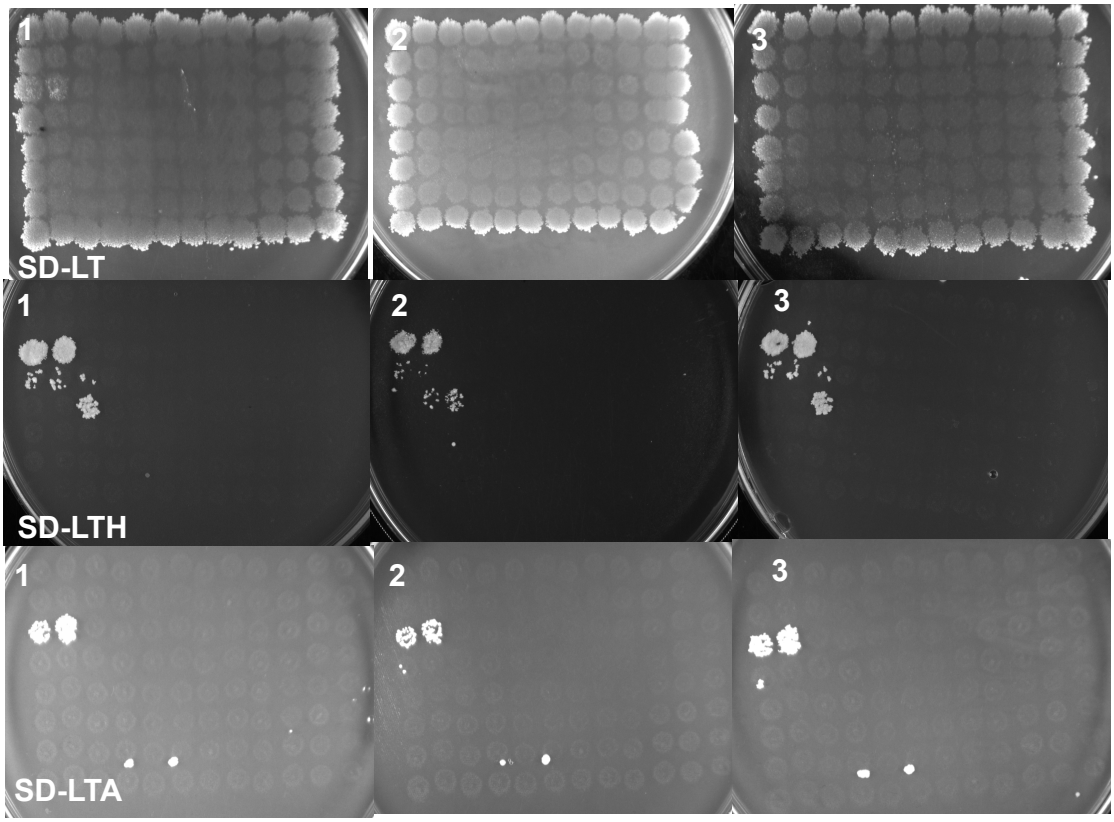


Figure 4.7 - The Y2H assay is reproducible with low background. Three independent mating reactions were performed on YPDA plates and plates were then printed on to selective media (SD-LT, SD-LTH and SD-LTA), cleaned and growth of colonies scored. SD-LTA picks up fewer interactions than SD-LTH as *ADE2* is a far more stringent selective reporter gene than *HIS3*, hence only very strong interactions were picked up on the SD-LTA plates. Growth on SD-LT plates indicate successful mating events between haploid cells.

This screen has a low background and interactions are reproducible and easy to identify. The next check was if this screen was reliably picking up known interactors of the JAZs. Throughout the assay controls were employed to ensure the correct media lacked the correct components and was therefore selective (Appendix 4).

4.2.3.1.1 JAZ proteins interact with known interactors in this Y2H screen

JAZ1, 2, 3, 4, 5, 6, 7, 8, 10 and 11 were checked for binding to the MYC TFs (MYC2, 3 and 4) on both selective media (SD-LTH and SD-LTA) (Fig.4.8). As can be seen, all JAZs screened bind to all three MYC proteins. Previous research supports our assay results, a unidirectional Y2H screen of JAZ:DB against MYC2:AD or MYC3:AD or MYC4:AD has previously found all JAZs (apart from

JAZ4 and JAZ7) to interact with MYC2, all JAZs apart from JAZ4 interact with MYC3 and all JAZs interact with MYC4 in yeast (Fernández-Calvo *et al.,* 2011)

4.2.3.1.1.1 MYC2

Subsequent Y2H screens have found MYC2 is able to interact with JAZ7 when MYC2 is in a BD vector and JAZ7 in an AD vector (Sasaki-Sekimoto *et al.,* 2014). Moreover, a separate Y2H screen found MYC2 could interact with all JAZs in an Y2H screen with the same unidirectional screen as employed in this section (Cheng *et al.,* 2011). *In-vivo* studies confirmed the MYC2 Y2H binding results, furthermore JAZ4 was also found to be binding to MYC2 in protein pull-down assays (Chini *et al.,* 2009).

4.2.3.1.1.2 MYC3 and MYC4

Moreover, a separate screen has shown MYC3 to weakly bind JAZ4, again in Y2H in the same direction as this screen (MYC:AD and JAZ:DB) (Goossens *et al.,* 2015). Although there are small discrepancies between these results, it gives an overall picture that MYC2, MYC3 and MYC4 bind most JAZs consistent with results shown here.

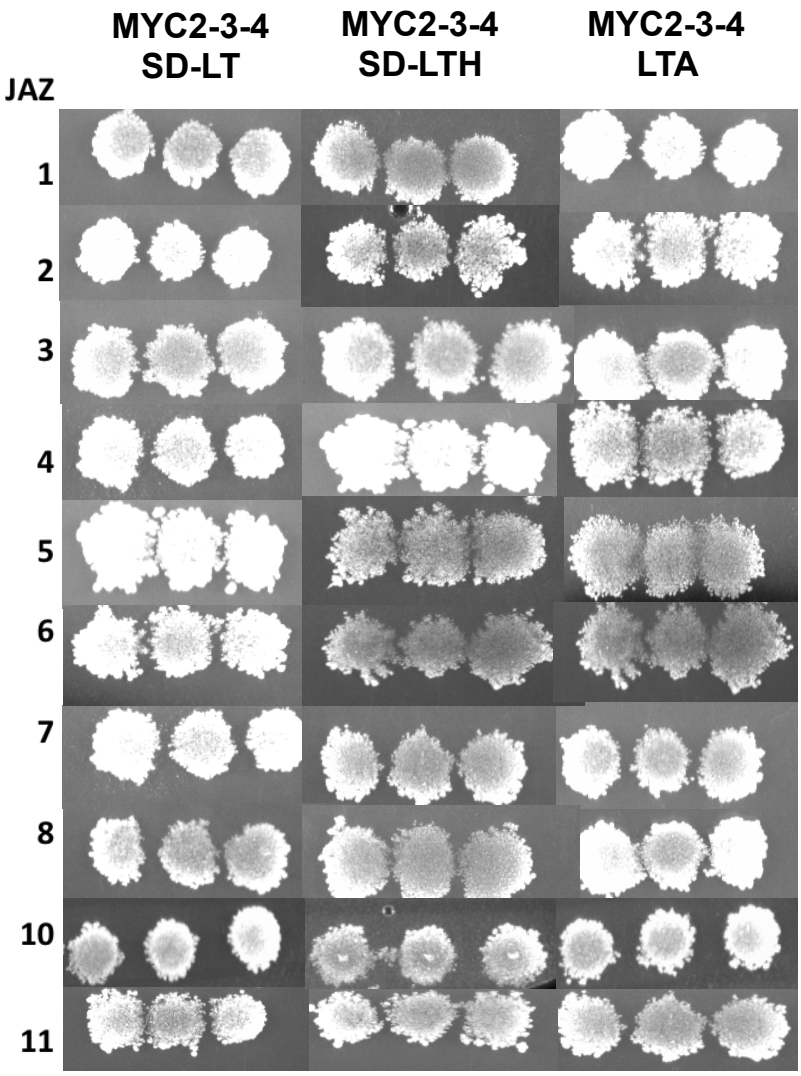


Figure 4.8 – MYCs2/3/4 are widely bound by JAZ proteins. JAZ DBs were screened against MYC2/3/4 ADs. Three independent mating reactions were performed on YPDA plates and plates were then printed on to selective media (SD-LT, SD-LTH and SD-LTA), cleaned and growth of colonies scored. SD-LTA picks up fewer interactions than SD-LTH as *ADE2* is a far more stringent selective reporter gene than *HIS3*, hence only very strong interactions were picked up on the SD-LTA plates. Growth on SD-LT plates indicate successful mating events between haploid cells.

4.2.3.2 JAZ6 binds to several defence and circadian clock related proteins

After confirming the screen was reliable and comparable to published data, the screen was used to investigate interactors of the JAZ6 protein against 1956 of the TF library (Pruneda-Paz *et al.,* 2014). All repeatable interactors of JAZ6 can be found in Table 4.1 and full TFs included in this screen are in Appendix 5, it

was found that JAZ6 interacts with several key TFs involved in defence and the circadian clock.

Table 4.1 - JAZ6 interacts with several key TFs involved in defence and the circadian clock. TF information including AGI number, name and well location are outlined. Growth scores for each interaction of SD-LTH and SD-LTA were recorded. The scoring system can be found in Chapter 2, in brief, 0 (red) = no growth, 1 (yellow)= very little growth, 2 (light green)= some growth and 3 (medium green)= lots of growth. Results are representative of three biological replicates per interaction. Well numbers highlighted in blue corresponds to what can be seen in Fig.4.9.

Gene identifier (AGI)	Gene name	Original location	SD-LTH	SD-LTA
AT1G67260	**TCP1**	U01-D12	2	0
AT1G35560	**TCP23**	U01-E02	2	0
AT5G56840	**UK MYB**	U02-D05	2	0
AT1G72450	**JAZ6**	U03-A03	3	1
AT1G69690	**TCP15**	U03-D12	2	0
AT1G58100	**TCP8**	U07-C02	2	0
AT3G27940	**LBD26**	U07-C11	1	0
AT1G48500	**JAZ4**	U08-C07	1	0
AT3G02150	**PTF1**	U09-E02	1	0
AT4G23860	**AT4G23860**	U09-E11	1	0
AT4G17880	**MYC4**	U13-A05	3	3
AT5G46760	**MYC3**	U13-A07	3	3
AT5G65640	**bHLH093**	U13-B01	1	0
AT1G01260	**JAM2**	U15-A02	2	0
AT2G26960	**MYB81**	U15-A11	2	0
AT1G69540	**AGL94**	U15-B12	1	0
AT3G45150	**TCP16**	U15-D12	1	0
AT5G17810	**WOX12**	U15-E12	2	1
AT2G33880	**HB-3**	U15-F01	1	0
AT1G28470	**NAC010**	U16-A10	1	0
AT5G67450	**ZF1**	U18-A02	1	0
AT5G51910	**TCP19**	U18-C02	2	0
AT1G32640	**MYC2**	U19-B01	3	3
AT3G20770	**EIN3**	U19-B12	1	0
AT3G22170	**FHY3**	U21-A11	2	2

In total, 25 repeatable interactions were found between JAZ6 and 1956 TFs (Table 4.1). Examples of positive interactors of JAZ6 can be seen in Fig.4.9. Interactions, which repeated more than once on SD-LTH plates, were deemed as true interactions. More interactions were observed on SD-LTH than on SD-LTA due to the Ade reporter being more stringent than the His reporter. Those, which interacted while on SD-LTA, were therefore likely to be stronger interactions.

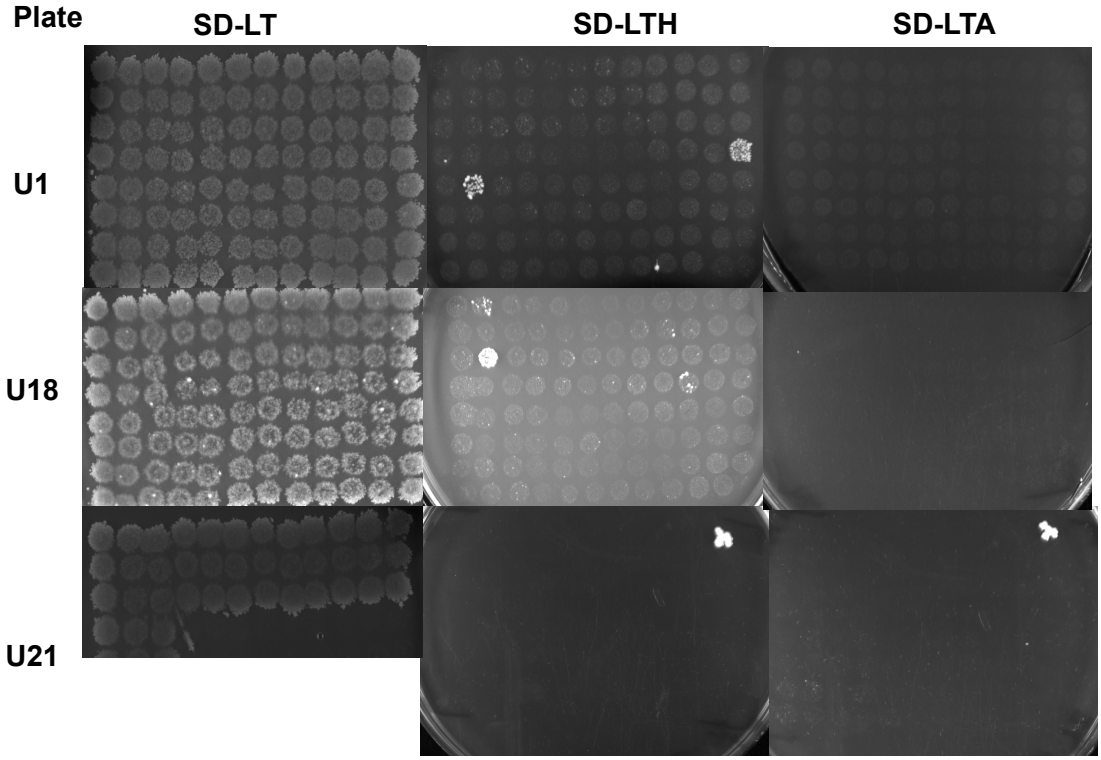


Figure 4.9 – JAZ6 interaction examples. A display of how the interactions were scored based on their colony formation on both selective medias (SD-LTH and SD-LTA) alongside SD-LT mating control plates. Plates U1, U18 and U21, are displayed, plate U1 shows a positive interaction between JAZ6 and TCP1 and TCP23, plate U18 shows a positive interaction between JAZ6 and both TCP19 and ZF1 and U21 displays a strong interaction between JAZ6 and FHY3.

4.2.3.2.1 Other JAZ proteins cannot bind to TFs which JAZ6 can

JAZ6 interacted with 25 TFs in the Y2H assay (Table 4.1). To investigate if these interactions were specific to JAZ6 or if they also occurred with other JAZ proteins a screen was performed against the 1956 TF library using a pool of JAZ proteins (DB:JAZs: 1, 2, 3, 4, 5, 7, 8, 10 and 11). This pool did not contain JAZ6 so any interactions detected in the TF library against this pool are not specific to JAZ6 and are bound by other JAZs.

The full TF list included in this screen can be found in Appendix 5 and positive interactors can be found in Table 4.2. In total, 41 proteins from the TF library

were shown to interact with the mixed JAZ pool (Table 4.2). This is greater than the number of interactors observed with JAZ6 alone (25), which is expected as a pool of nine JAZ proteins will have more interactions than one individual JAZ protein.

Table 4.2 – A pool of JAZ DBs (JAZ1, 2, 3, 4, 5, 7, 8, 10 and 11) interacted with many known and unknown protein targets. TF information including AGI number, name and well location are outlined. Growth scores for each interaction of SD-LTH and SD-LTA are recorded. The scoring system can be found in Chapter 2, in brief, 0 (red) = no growth, 1 (yellow)= very little growth, 2 (light green)= some growth and 3 (medium green)= lots of growth. Results are representative of three biological replicates per interaction.

Gene identifier (AGI)	Gene name	Original location	SD-LTH	SD-LTA
AT1G67260	**TCP1**	U01-D12	1	0
AT1G35560	**TCP23**	U01-E02	2	0
AT1G72450	**JAZ6**	U03-A03	1	0
AT2G47460	**MYB12**	U04-A02	1	0
AT3G23240	**ERF1**	U04-A08	1	1
AT3G50060	**MYB77**	U04-B05	1	0
AT1G58100	**TCP8**	U07-C02	2	0
AT2G45680	**TCP9**	U07-F11	2	0
AT1G30135	**JAZ8**	U10-F01	1	0
AT5G46830	**NIG1**	U11-F01	2	0
AT3G54990	**SMZ**	U11-G01	1	0
AT4G17880	**MYC4**	U13-A05	3	3
AT5G46760	**MYC3**	U13-A07	3	3
AT5G09460	**AT5G09460**	U14-D07	1	0
AT1G31760	**SWIB**	U14-G12	2	0
AT3G02860	**ARS1**	U14-H05	1	0
AT1G01260	**JAM2**	U15-A02	3	3
AT1G24260	**SEPELLATA3**	U15-A05	1	0
AT2G33720	**AT2G33720**	U15-A07	1	0
AT2G26960	**MYB81**	U15-A11	1	0
AT5G65310	**HB5**	U15-B01	2	0
AT5G47670	**NF-YB6**	U15-B05	1	0
AT1G69540	**AGL94**	U15-B12	1	0
AT4G18960	**AG**	U15-C12	1	0
AT4G14490	**AT4G14490**	U15-D02	1	0
AT3G45150	**TCP16**	U15-D12	1	0
AT3G18550	**BRC1**	U15-E01	1	0
AT3G12910	**NAC UK**	U15-E02	1	0
AT2G33880	**HB-3**	U15-F01	1	0
AT1G65620	**AS2**	U15-G01	1	0
AT1G68810	**ABS5**	U15-G04	1	0
AT3G49760	**bZIP5**	U15-H01	1	0
AT5G08130	**BIM1**	U15-H02	1	1
AT1G70920	**HB18**	U15-H03	1	0
AT5G51910	**TCP19**	U18-C02	1	0
AT3G50510	**LBD28**	U18-C06	1	1
AT4G32880	**HB-8**	U18-D11	2	2
AT1G32640	**MYC2**	U19-B01	3	3
AT5G23280	**TCP7**	U20-B12	1	0
AT5G08330	**TCP21**	U20-D12	1	0
AT2G27110	**FRS3**	U20-G07	2	1

The reliability of these results was affirmed when MYC2, MYC3 and MYC4 were again shown to repeatedly bind JAZ6 and the pool of mixed JAZs on both selective medias (Fig.4.10). Moreover, JAZ6 was also shown to bind to several other JAZ proteins (JAZ4 and JAZ6), as were the mixed JAZs (JAZ6 and JAZ8), this is comparable to previous research, which has shown the JAZ family to be highly interconnected with its own regulatory interactome consisting of many JAZ:JAZ protein homo- and heterodimers (Chini *et al*., 2009). Additionally, JAZ6 has been shown to bind to itself in both AD/BD orientations in a previous Y2H screen (Chung *et al.,* 2009).

This screen identified three sets of TFs within the 1956 library, those that bound JAZ6 uniquely, those that bound both JAZ6 and the mixed JAZs and those that were bound by only the pooled JAZs (Fig.4.10). 12 TFs were exclusively bound by JAZ6, the mixed JAZ pool exclusively bound 28 TFs and both JAZ6 and the mixed JAZs bound 13 TFs (Fig.4.10).

4.2.3.2.2 Pooling DB proteins reduces the number of interactors

Given the relatively small number of mixed JAZ targets compared to JAZ6 targets alone it can be hypothesized that pooling the JAZ DBs and performing the screen may have lowered the sensitivity of the screen and missed some interactions between individual JAZs and the TF library. Theoretically, assuming all JAZ proteins had approximately the same number of binding partners, it would be assumed as JAZ6 exclusively bound 12 proteins the mixed JAZ pool containing nine JAZs would bind 9 multiplied by 12, so 108 proteins in total. To check if the pooling the JAZs had caused interaction to be missed a JAZ5:DB was individually screened against 768 proteins in the TF library. Four proteins in this group repeatedly interacted with JAZ5. Two of these proteins had also been shown to bind with the mixed JAZ pool but the JAZ5-DB bound two proteins which did not interact with the pooled JAZs. Of the 768 proteins screened, 25 had been shown to interact with the mixed JAZ pool only and not

the JAZ5 DB. Results of this screen can be found in Appendix 6. As such it can be assumed that by pooling the JAZ DBs 50% interactions have been missed. However, due to the high false positive rate reported when employing Y2H it may be that this was not due to pooling JAZ proteins and was just a limitation of the technique. For example two large-scale Y2H screens were performed following the same methodology and only 30% of the positive interaction were shared between both screens (Ito *et al.*, 2001). Had time and resources allowed each individual JAZ-DB would have been screened against the full TF library. It may be that the overall protein levels in the pooled JAZ set was lower than when individual JAZs were screened, hence certain interactions were missed, previous research has confirmed lower protein concentrations in Y2H can miss weaker interactions (Vidalain *et al.,* 2004).

A subset of interactions exclusive to either the mixed JAZ pool or the JAZ6 DB and also shows shared interactors between the two DB sets can be seen in Fig.4.10. All proteins found to be interactors of JAZ6 or/and the mixed JAZ pool were included in this subset. TFs that interacted exclusively with the mixed JAZ pool were included so individual JAZ binding partners could be elucidated. Any JAZ proteins were not carried into the subset list as previous research has thoroughly investigated the protein interactions between the JAZ family proteins using Y2H (Chini *et al*., 2009). This gave a total of 50 proteins in the subset; MYC2, MYC3 and MYC4 were included in this 50 as positive controls.

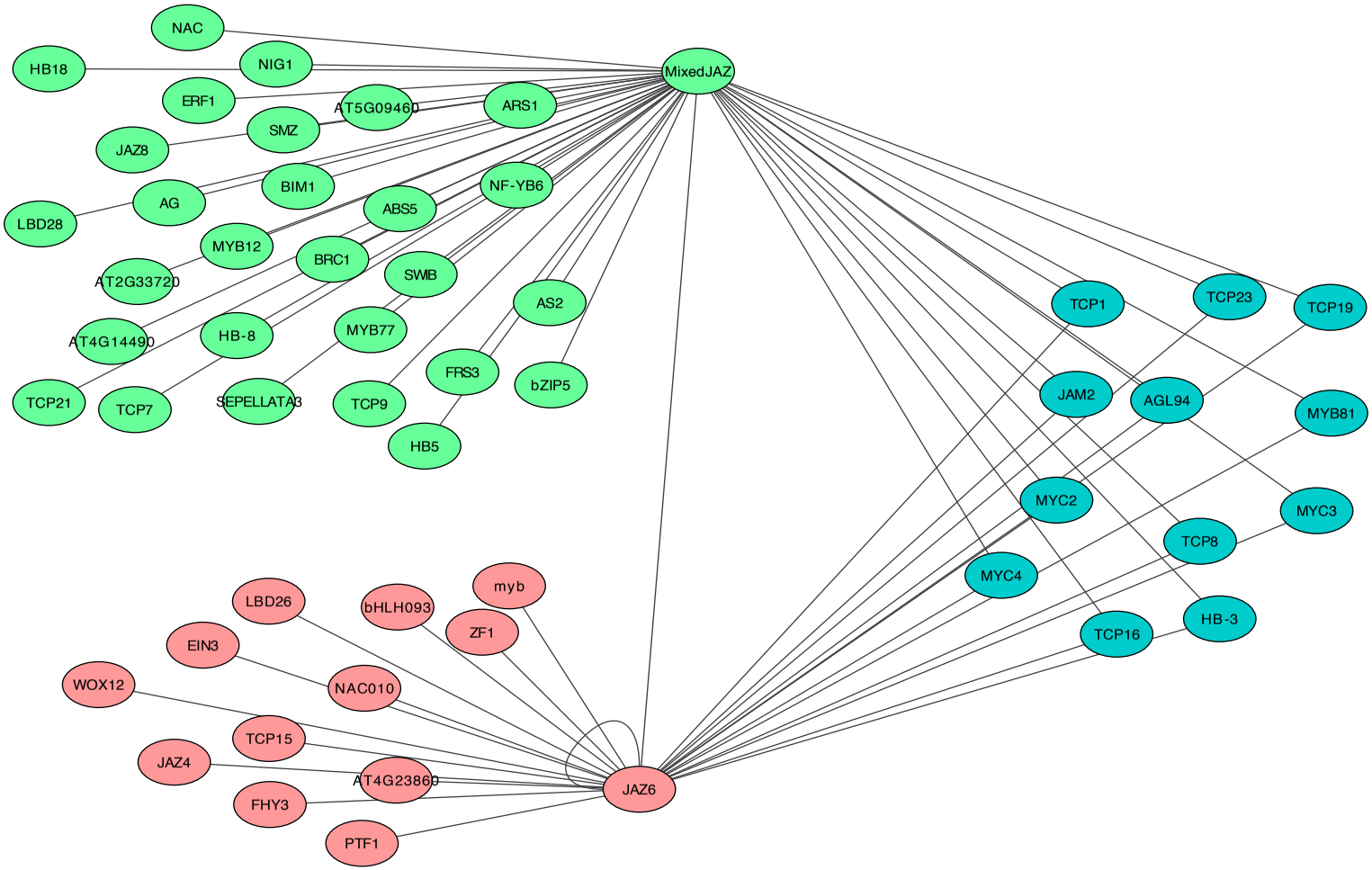


Figure 4.10– Protein to protein interaction network model of JAZ6 DB and mixed JAZ DB against the 1956 TF library. Exclusive JAZ6 binding partners can be seen in salmon, exclusive Mixed JAZ interactors can be seen in green and shared interactions between both JAZ6 and the mixed JAZ pool can be seen in turquoise (visualized using Cytoscape v2.8.3 (Shannon *et al.,* 2003))

4.2.3.2.3 JAZ5, JAZ6, JAZ7 and JAZ10 bind to an array of TFs

Plants with reduced expression in *JAZ5, JAZ7 and JAZ10* maintained a difference in resistance levels to *B. cinerea* when inoculated at dawn compared to night, the same as observed in Col-0. However, plants with reduced expression in *JAZ5, JAZ6* and *JAZ10* showed no difference in resistance levels to *B. cinerea* when inoculated at dawn compared to night and neither did a single mutant with reduced *JAZ6* levels only (Fig.4.2).

To identify how JAZ6 could be influencing the defence response differently to JAZ5, JAZ7 or JAZ10, the JAZ5, JAZ6, JAZ7 and JAZ10 DBs were screened individually for protein-protein interactions against the targets list referenced below (Table 4.3).

Table 4.3 – JAZs 5/6/7/10 individually bound many targets previously identified by screening pooled and JAZ6 DBs. TF information including AGI number and name are outlined. Results are representative of three biological replicates per interaction. Results can be compared with those previously recorded for JAZ6 (pink) and the pooled JAZs (mix – pink) in the 'previous screen' column, those that interacted with both JAZ6 and the mixed pool are referred to as 'shared' in this column. 41 interactions matched previous results whereas 9 did not.

Gene identifier	Target list	JAZ5	JAZ6	JAZ7	JAZ10	Previous screen	Matching previous?
AT5G56840	**myb like**	-	yes	-	-	jaz6	yes
AT1G69690	**TCP15**	yes	yes	yes	yes	jaz6	no
AT3G02150	**PTF1**	yes	yes	-	-	jaz6	no
AT4G23860	**AT4G23860**	-	yes	-	-	jaz6	yes
AT5G65640	**bHLH093**	-	yes	yes	-	jaz6	no
AT5G17810	**WOX12**	-	yes	-	-	jaz6	yes
AT1G28470	**NAC010**	-	yes	-	-	jaz6	yes
AT5G67450	**ZF1**	-	yes	-	-	jaz6	yes
AT3G20770	**EIN3**	-	yes	-	-	jaz6	yes
AT3G22170	**FHY3**	-	yes	-	-	jaz6	yes
AT2G47460	**MYB12**	-	-	-	-	mix	yes
AT3G23240	**ERF1**	yes	-	-	-	mix	yes
AT3G50060	**MYB77**	yes	yes	yes	yes	mix	no
AT2G45680	**TCP9**	yes	yes	yes	yes	mix	no
AT5G46830	**NIG1**	yes	-	-	yes	mix	yes
AT3G54990	**SMZ**	-	-	-	yes	mix	yes
AT5G09460	**AT5G09460**	-	-	-	-	mix	yes
AT1G31760	**SWIB**	-	-	-	-	mix	yes
AT3G02860	**ARS1**	yes	-	yes	-	mix	yes
AT1G24260	**SEPELLATA3**	yes	-	-	yes	mix	yes
AT2G33720	**AT2G33720**	yes	-	yes	yes	mix	yes
AT5G65310	**HB5**	-	-	-	-	mix	yes
AT5G47670	**NF-YB6**	yes	-	-	-	mix	yes
AT4G18960	**AG**	yes	-	yes	yes	mix	yes
AT4G14490	**AT4G14490**	yes	-	-	-	mix	yes
AT3G18550	**BRC1**	yes	-	yes	yes	mix	yes
AT3G12910	**NAC UK**	-	-	yes	yes	mix	yes
AT1G65620	**AS2**	-	-	yes	yes	mix	yes
AT1G68810	**ABS5**	-	-	yes	-	mix	yes
AT3G49760	**bZIP5**	-	-	yes	-	mix	yes
AT5G08130	**BIM1**	yes	-	-	yes	mix	yes
AT1G70920	**HB18**	yes	-	-	-	mix	yes
AT3G50510	**LBD28**	yes	-	yes	yes	mix	yes
AT4G32880	**HB-8**	-	-	-	yes	mix	yes
AT5G23280	**TCP7**	yes	yes	-	yes	mix	no
AT5G08330	**TCP21**	yes	yes	-	yes	mix	no
AT2G27110	**FRS3**	yes	yes	yes	-	mix	no
AT1G67260	**TCP1**	yes	yes	yes	yes	shared	yes
AT1G35560	**TCP23**	yes	yes	-	yes	shared	yes
AT1G58100	**TCP8**	yes	yes	yes	yes	shared	yes
AT4G17880	**MYC4**	yes	yes	yes	yes	shared	yes
AT5G46760	**MYC3**	yes	yes	yes	yes	shared	yes
AT1G01260	**JAM2**	yes	yes	yes	yes	shared	yes
AT2G26960	**MYB81**	yes	yes	yes	yes	shared	yes
AT1G69540	**AGL94**	yes	yes	yes	yes	shared	yes
AT3G45150	**TCP16**	yes	yes	yes	yes	shared	yes
AT2G33880	**HB-3**	yes	yes	yes	-	shared	yes
AT5G51910	**TCP19**	yes	yes	-	yes	shared	yes
AT1G32640	**MYC2**	yes	yes	yes	yes	shared	yes

Table 4.3 compares results obtained from the previous two screens using pooled JAZs (mixed JAZ) and JAZ6 alone against the full 1956 TF library to the results obtained when screening individual JAZ DBs (JAZ5, JAZ6, JAZ7 and JAZ10) against the TF subset list. Several proteins from the 'TF subset list' did not interact with any of the four JAZs, this is expected as the subset list included proteins which exclusively bound with a pool of mixed JAZs, which contained more than the four screened. Of the 49 proteins screened, 41 (82%) of the results obtained match the previous mixed JAZ and JAZ6 results. However, eight proteins (16%) showed different results in this individual JAZ5/6/7/10 screen compared to the mixed JAZ pool and JAZ6 screens.

Three of these eight proteins (TCP15, PTF1 and bHLH093) were previously shown to bind to JAZ6 only and not the mixed JAZ pool, however in this screen these proteins also bound one or more of the JAZ5/7/10. It is possible that by pooling the JAZs in the mixed JAZ screen we missed these individual interactions (as has been demonstrated in Appendix 6). Or it is possibly due to the high false negative recorded in Y2H assays (Ito *et al.*, 2001). The remaining five proteins were previously shown to only bind to the mixed JAZ pool and not JAZ6, however in this TF subset list screen these five bound to JAZ6. Given the same DB of JAZ6 was used in this screen as the previous screen and the same methods were used, how could these mismatches occur? Looking at the individual proteins, it can be seen three of this group of five are in the Teosinte Branched Cycloidea and PCF (TCP) family (TCP9, 7 and 21).

It was previously shown in the extensive Y1H screens carried out by PRESTA (such as that employed in Breeze *et al.,* 2011 and Hickman *et al.,* 2013) that TCPs can promiscuously binding to a wide variety of promoters, it is not known whether this is due to a true biological mechanism, a result of false positives in the Y1H screen or these proteins being "sticky". The concept of "sticky" proteins was outlined in Vidalain *et al.,* (2004), it was stated that there are certain AD proteins, which have a structure that allows them to bind to many other proteins in Y2H. Given the high level of TCP binding to JAZ5/6/7/10 and the mixed JAZ pool, the TCPs may be "sticky". It could therefore be speculated that

the reason these TCPs give unrepeatable results is due to their "stickiness" yielding unreliable results, however further work *in-planta* needs to be carried out to confirm the TCPs are not truly binding the JAZ proteins employed in this screen.

The remaining two proteins, which have shown variable results between the screens, are FRS3 and MYB77, both of which bound JAZ6 in the targets list screen but not in the full TF library screen. The FRS3 (FAR1-related sequence 3) TF has been involved in light perception and is bound by JAZ3 in Y2H; however, JAZ6 was not included in this screen (Arabidopsis Interactome Mapping Consortium, 2011). MYB77 (myb domain protein 77) has previously been shown in Y2H to bind to both TPR1 (topless-related protein 1) and TPL (topless) (Causier *et al.,* 2011). Given both these proteins seem closely linked with the JAZ family; it seems unlikely they are spontaneous false positives. JAZ6 could be interacting with them both, however, given these interactions were not previously detected for unknown reasons further repeats are required to confirm this.

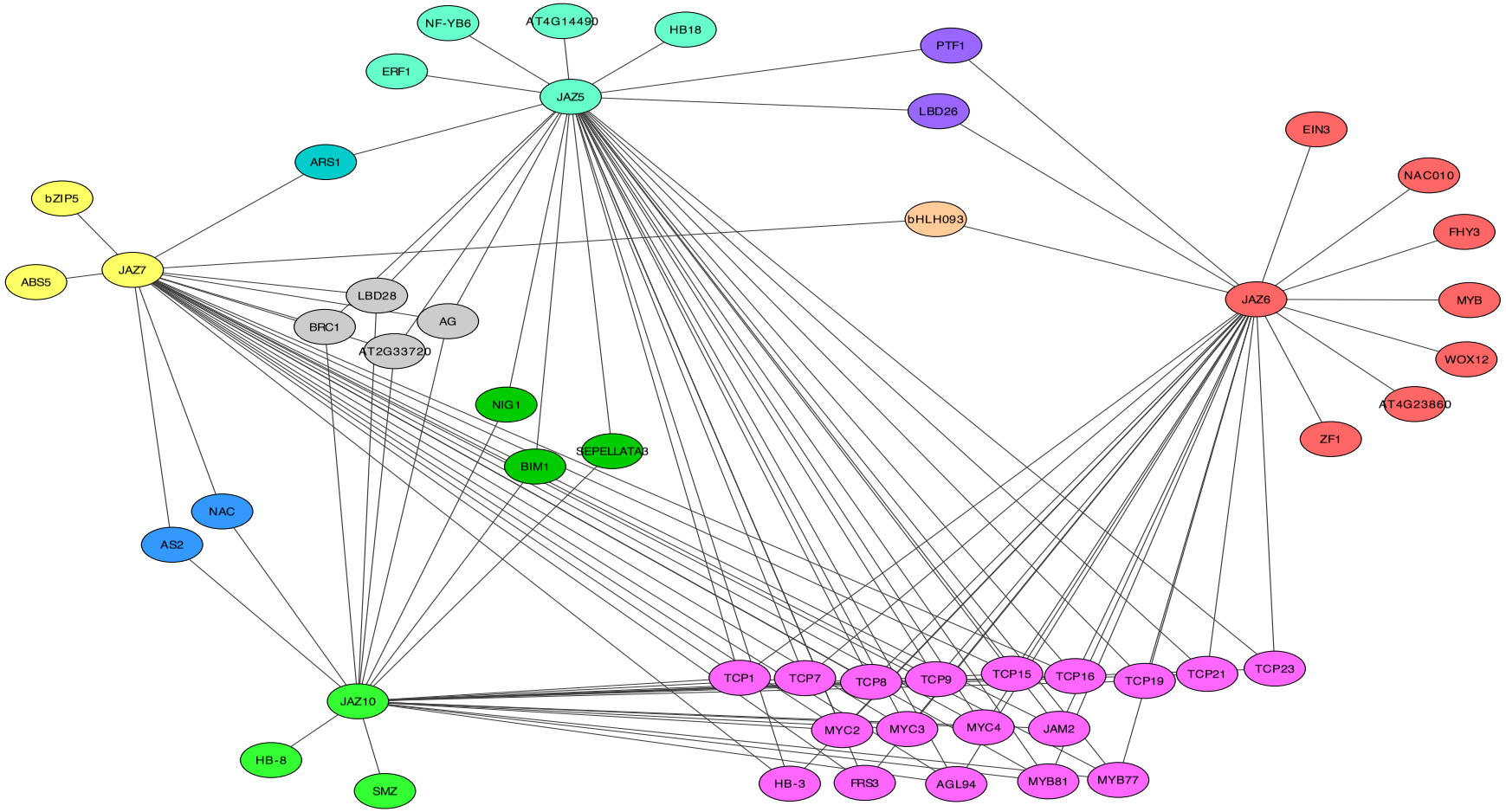


Figure. 4.11 – Protein to protein interaction network model of JAZ5/6/7/10 DBs against the target list of TFs. Exclusive JAZ5 targets (mint), exclusive JAZ6 binding partners (salmon), exclusive JAZ7 interactors (yellow), exclusive JAZ10 interactors (bright green). TFs with high binding levels to several JAZs can be seen in pink (visualized using Cytoscape v2.8.3 (Shannon *et al.,* 2003))

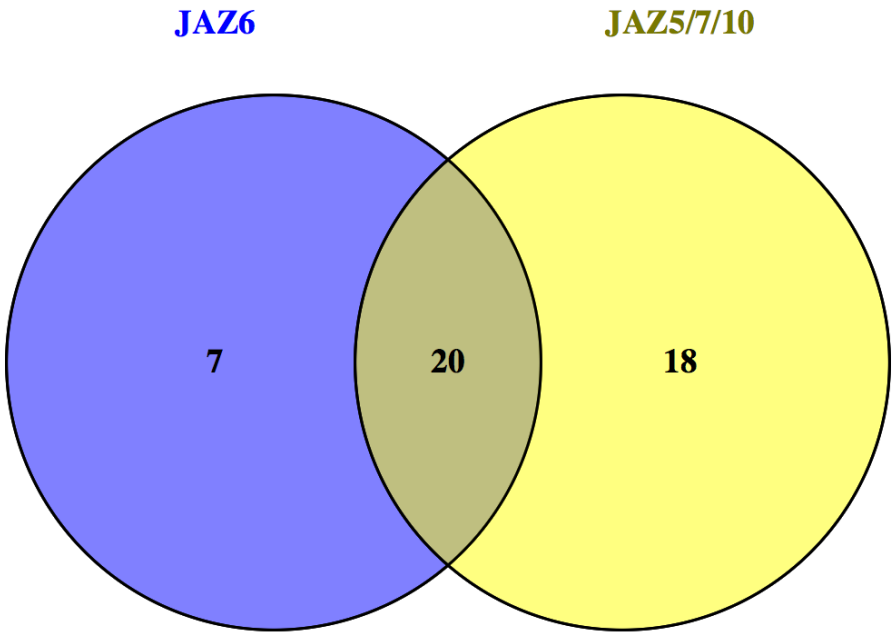


Figure 4.12 –Interactions between individual JAZ6 and TF ADs compared with interactions between individual JAZ5, 7 or/and 10 and TF ADs. Venn diagram displaying interactions discovered when JAZ6 was individually screened against 768 of the TF library (replicates = 3) (blue) and those interactions discovered when the either individual JAZ5, 7 or 10 were screened against the same 768 of the TF library (yellow). The green shade represented the shared interactions between the two groups.

4.2.3.3 JAZ proteins interact with circadian clock and JA signalling proteins

In total 15 proteins were found to have exclusive interactions with either JAZ5 (n = 4), JAZ6 (n =7), JAZ7 (n =2) or JAZ10 (n =2) and 18 proteins (including the three MYCs) were shown to interact with all four individual JAZ proteins screened (Fig.4.11). 20 proteins were shown to be capable of binding both JAZ6 and at least one of either; JAZ5 or JAZ7 or JAZ10 (Fig 4.12).

Three MYCs bound with all JAZ proteins and these expected to bind to all the JAZs screened given previous research (Fernández-Calvo *et al.,* 2011). Given that these TFs bind many JAZs and are not specific to JAZ6, based on this screen alone, it is unlikely these are directly involved in the differential resistance observed to *B. cinerea* depending on time of day of inoculation.

Exclusive JAZ6 interactors were identified as targets for linking the circadian

clock to the defence response. As such, these interactors were further mined; first it was ensured that these proteins were expressed in adult leaves (the tissue used during *B. cinerea* inoculations), then any previous phenotype of plants mutated in the expression of these proteins and inoculated with *B. cinerea* were recorded, furthermore any previous Y2H data (obtained from BioGrid Stark *et al.,* (2006)) showing interactions between the target list TFs and the JAZ family (JAZs), MYCs 2, 3 or 4 (MYCs), TPL or TPL-like (TPLs) or circadian clock proteins were documented (Table 4.4). In doing this, it can be seen genes such as *EIN3* are expressed in adult leaves, the proteins they encode for have previously been shown to interact with circadian and/or JA pathway related proteins and/or mutants of this gene have shown a phenotype when mutated and inoculated with *B. cinerea* (Table 4.4).

Table 4.4 – Information on TFs that interacted with JAZ6. Information includes AGI number, Gene name, interactions recorded within this chapter, expression information (ePlant (https://bar.utoronto.ca/eplant/) (Fucile *et al.,* 2011)) (LE – leaf 7 expression), any previous phenotype recorded when infected with *B. cinerea* (WL – white light) and any previous Y2H results (BioGrid). Under LE – lowly is defined as low expression, below 0.5 on a Log2 Ratio scale.

Gene identifier (AGI)	Target list	Y2H				Expressed in adult leaves	Previous phenotype with Botrytis	DE-TF in microarray	ChIP			Previous Y2H results (BioGrid)			
		JAZ5	JAZ6	JAZ7	JAZ10				TOC1	PRR5	CCA1	JAZs	MYCs	TPLs	Clock proteins
AT5G56840	**myb like**	-	yes	-	-	-	-	UPUP18	-	-	-	-	-	-	-
AT4G23860	**AT4G23860**	-	yes	-	-	yes	-	-	-	-	-	-	-	-	-
AT5G17810	**WOX12**	-	yes	-	-	-	-	-	-	-	-	-	-	-	-
AT1G28470	**NAC010**	-	yes	-	-	lowly	-	-	-	-	-	-	-	-	-
AT5G67450	**ZF1**	-	yes	-	-	yes	-	-	-	-	-	-	-	-	-
AT3G20770	**EIN3**	-	yes	-	-	yes	ein3-1 more susc. Alonso 2003	-	-	-	-	1	2, 3 and 4	-	-
AT3G22170	**FHY3**	-	yes	-	-	yes	-	-	-	-	-	-	-	-	CCA1/LHY/HY5

4.2.4 *ein3-1* loses time of day dependent differences in response to *B. cinerea* inoculations at subjective dawn or subjective night

Ethylene insensitive 3 (EIN3) is expressed in adult leaves and plays a key role in plant defence by mediating the cross talk between JA and ET regulation upon necrotrophic pathogen attack (Song *et al.,* 2014). In Table 4.4 it can be seen EIN3 interacts with JAZ6 and did not interact with JAZ5/7/10 in the individual JAZ screen nor did EIN3 interact with the JAZs when pooled (Mixed JAZs). EIN3 has previously been shown to interact with JAZ1/3/9 *in-planta* (Zhu *et al.,* 2011). JAZ1 represses both EIL1 and EIN3 by directly binding and recruiting HDA6 and it has been inferred that JAZ3 and JAZ9 functions in a similar manner (Zhu *et al.,* 2011). Presumably, if JAZ6 does interact with EIN3 *in-planta* it will also recruit such HDA's to repress EIN3 however this needs further *in-planta* confirmation.

EIN3 has previously been shown to be a positive regulator in the defence response against *B. cinerea*, with *ein3-1* mutants showing significantly enhanced susceptibility to *B. cinerea* (infection time not stated) (Zhu *et al.,* 2011). JAZ6 binding to and regulating such a crucial TF would explain the powerful effects losing *JAZ6* expression has on defence against *B. cinerea*.

To test the involvement of EIN3 in this circadian regulated defence pathway against *B. cinerea,* an *ein3-1* mutant was screened for differences in susceptibility when inoculated at dawn compared to night (Fig 4.13).

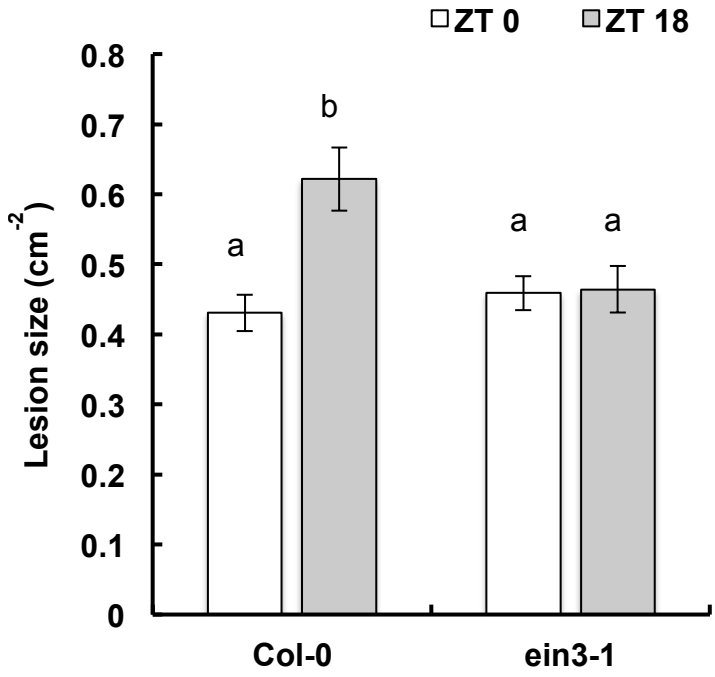


Figure 4.13 – *ein3-1* loses time of day dependent differences in response to *B. cinerea* inoculations at subjective dawn or subjective night. Col-0 and *ein3-1* leaves from four-week-old plants were inoculated with *B. cinerea* under LD conditions at dawn (ZT0) or night (ZT18) and lesion area measured at 72 hpi. Data shown are mean lesion sizes ± SEM of wild-type (Col-0) leaves versus *ein3-1* mutants (n ≥30 per line and inoculation time). This is representative of one experiment. Two-way ANOVA was used to test whether lesion size was significantly different between *ein3-1* inoculated at dawn versus night and Col-0 leaves inoculated at dawn versus night, with a *p*-value threshold of 0.05 applied.

Contradictory to previous results (Zhu *et al.,* 2011), *ein3-1* showed decreased susceptibility to *B. cinerea* inoculated when compared to Col-0. The loss of EIN3 lead to a loss in time-dependent defences to *B. cinerea* (Fig 4.13). This suggests that the protein interaction between JAZ6 and EIN3 contributes to circadian regulated defences against *B. cinerea*.

4.3 Discussion

4.3.1 *JAZ6* links the circadian clock to the defence response against *B. cinerea*

In this study it was found that where a *jaz5/7/10* mutant maintained the same phenotype as Col-0, a *jaz5/6/10* mutant displayed a difference to Col-0 and showed no differences in lesion size between dawn and night *B. cinerea* inoculations under LL or LD conditions (Fig.4.1). Moreover, the *jaz5, jaz10* and *jaz5/10* mutants exhibited the same phenotype as Col-0, whereas a single *jaz6* mutant displayed equal levels of immunity whether inoculated at dawn or night under LD conditions (Fig.4.2). Given this, it was hypothesized *JAZ6* was linking the defence response to the circadian clock.

Two central circadian oscillators TFs (PRR5 and PRR7) directly bind the *JAZ6* promoter region, showing it is most likely under direct circadian regulation (Appendix 1)(Liu *et al.* (2013a); Nakamichi *et al.* (2012)). Furthermore, *JAZ6* has also been shown to display a circadian expression pattern under several different unstressed conditions and at different Col-0 developmental ages (both leaves and seedlings) with peak expression at ZT 3 (Fig.4.14) (Blasing *et al.*, 2005; Covington *et al.,* 2008). It is unknown at this stage if protein levels correspond with rhythmic transcript levels.

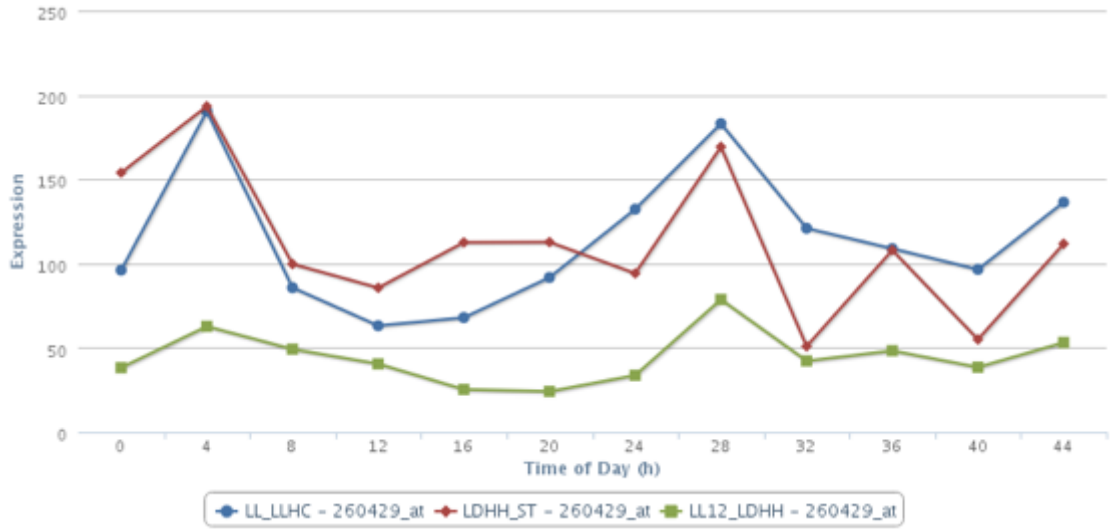


Figure 4.14 – *JAZ6* displays a circadian expression pattern under numerous growth conditions, with peak expression at ZT 4. LL_LLHC Seedlings grown on agar for 9-days under CL and cyclic hot:cold conditions. Seedlings were then placed in LL and constant temperature for 24 h and then harvested. LDHH_ST Plants were grown on soil under 12:12 LD conditions and kept at a constant temperature for 35 days - leaves were then harvested. LL12_LDHH Seedlings were grown on agar for 7-days under 12:12 LD conditions and kept at a constant temperature. Seedlings were then placed in LL and constant temperature for 24 h and then harvested. All experiments were carried out on Col-0 plants under a constant temperature of 22°C. Expression – RNA expression levels (log^2) (Blasing *et al.*, 2005; Covington *et al.,* 2008). Image from Diurnal (Mockler *et al.,* 2007).

JAZ6 expression has been shown to be upregulated in response to *B. cinerea* infection (inoculation time: ZT 6) (Fig.4.15) (Windram *et al.,* 2012). The greatest increase in expression levels of *JAZ6* in response to inoculation can be observed between 18-34 hpi (Fig.4.15).

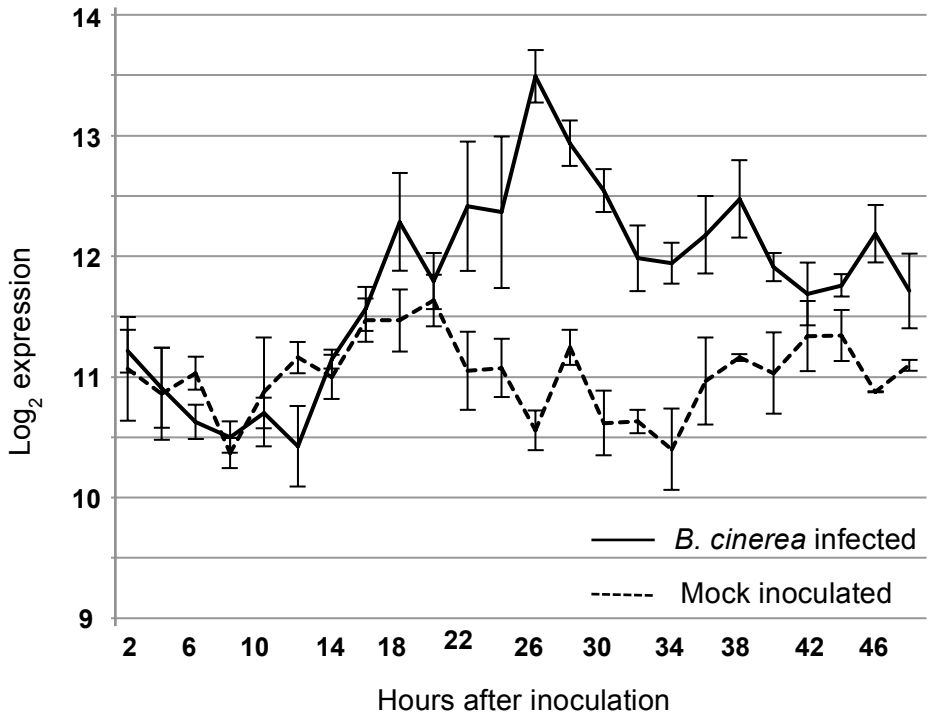


Figure 4.15 - *JAZ6* expression is transiently induced during *B. cinerea* infection. The expression of *JAZ6* is shown from the data of Windram *et al.* (2012). Arabidopsis leaves were inoculated with *B. cinerea* spores or mock-inoculated and sampled every 2 hours for 48 hours. The data shown are the mean of four biological replicates at each time point ± SEM. (Reproduced with permissions from Ingle *et al.,* 2015)

The rhythmic expression profile (Fig.4.14) and upregulation of the *JAZ6* transcript in response to *B. cinerea* inoculation (Fig.4.15) further validated the hypothesis that *JAZ6* was a link between the circadian clock and the defence response. The *jaz6* mutant was then further screened for susceptibility under LL conditions and again no difference was observed between dawn and night lesion sizes (Fig.4.5). Moreover, a *35S:JAZ6-OX* line also exhibited a difference to Col-0, in that is also lost any differences observed between lesions sizes formed from dawn and night inoculations (Fig.4.6).

However, given most of this evidence was based on phenotyping of knockout and overexpressor lines, this comes with its own set of challenges when investigating the role of proteins which form larger complexes. JAZ proteins form hetero- and homodimers with one another (Chini *et al.,* 2009) and with members of other families such as MYC-type and bHLH proteins (Geerinck *et al.,*

2010) to form larger complexes. Mutating the expression of one protein member in such complexes could have several inadvertent effects and mutating one gene may not directly cause the phenotypes observed. In the case of *JAZ6*, it could be that by altering the expression and therefore protein levels of this gene, this has altered the protein complex and allowed other protein members to bind to this complex. Therefore, the phenotypes observed may not be due to the altered *JAZ6* expression directly but may be due to other protein members binding in the complex.

The *JAZ6-OX* line in which *JAZ6* is a constitutively expressed and the *jaz6* knockout line both displayed the same phenotype of a constitutively high level of resistance to *B. cinerea* (Fig.4.6). There are several possibilities as to how these phenotypes may come about. Col-0 plants inoculated on a night display increased susceptibility compared to those inoculated at dawn; however, when *JAZ6* is knocked out this increased susceptibility is lost. This suggests JAZ6 exhorts its activity in response to night inoculations. If at night in Col-0 JAZ6 were repressing a negative regulator of the necrotrophic defence response such as MYC2 (Song *et al.,* 2014) then a *jaz6* mutant would be more susceptible to *B. cinerea* inoculation. However, a *jaz6* mutant displayed increased resistance at night suggesting the repressive action JAZ6 exhorts at night is on a positive regulator of the defence response (Fig.4.5/Fig.4.6). When JAZ6 is removed from this, the positive regulator is then either unbound so free to enhance the defence response independent of time of day, or the positive regulator is bound by a non-circadian protein which may exist in lower levels or have a weaker binding ability than JAZ6 so the positive regulator can still constitutively enhance the defence response.

4.3.2 JAZ proteins have redundant roles in the plant biotic defence pathway

Given the high levels of redundancy within the JAZ family (Thireault *et al.,* 2016) it is unexpected that reducing the expression of only *JAZ6* can confer such a distinctive phenotype.

Redundancy among the JAZ family has clearly been displayed in biotic defence responses against the hemibiotroph, *P. syringae.* Single knockouts of *JAZs 1-7, 10* and *12* displayed wild-type phenotypes against *P. syringae*, except *jaz10* which in agreement with Demianski *et al.,* (2012) displayed marginally increased levels of chlorosis (de Torres Zabala *et al.*, 2015). Whereas a double *JAZ5* and *JAZ10* knockout line displayed enhanced susceptibly, which was specific to this double mutant and other double JAZ mutations (*jaz3/5, jaz5/6* and *jaz3/10*) were screened and did not confer this phenotype (de Torres Zabala *et al.*, 2015). It therefore appears *JAZ5* and *JAZ10* act redundantly as positive regulators in the defence response against *P. syringae*; it has been shown the proteins do this by protecting the plant against the pathogen produced JA mimic, coronatine (COR) (de Torres Zabala *et al.*, 2015).

4.3.3 Individual JAZ proteins have distinctive roles in the plant biotic defence pathway

However, similar to *jaz6* several other individual JAZ mutants have also resulted in phenotypic differences in their biotic defence responses so it is likely the case that as well as acting redundantly individual JAZ proteins perform unique biological functions.

JAZ7, for example, is assumed to act individually. JAZ7 has never been shown to form hetero and homodimers with any other JAZ proteins (Chini *et al*., 2009) and has only been found to bind to very few TFs (MYC3/4) (Fernández-Calvo *et al.*, 2011) and JAM1 (Thatcher *et al.,* 2016). Single *JAZ7* mutant lines have conferred various phenotypes. A *JAZ7* knockout line has exhibited increased sensitivity to dark induced senescence (Yu *et al.,* 2016). Whereas a *jaz7-1D* line exhibiting constitutive overexpression of *JAZ7* exhibited increased sensitivity to JA, increased susceptibility to the hemibiotrophic pathogens, *F. oxysporum,* and *P. syringae* (Thatcher *et al.,* 2016). Given these factors it appears that *JAZ7* is a negative regulator of the plant defence response against both bacterial and fungal pathogens.

Moreover, a modified version of the JAZ1 protein (JAZ1 Δ 3A) with a mutated jas domain (which COI1 binds) exhibited a JA-insensitive phenotype and displayed increased susceptibility to *B. cinerea* and increased resistance to *P. syringae* (Thines *et al.,* 2007). The sensitivity to *B. cinerea* infection was attributed to the reduced degradation of JAZ1 and hence the reduced camalexin levels in this line.

A recently characterised thirteenth JAZ protein (JAZ13) was also shown to confer a phenotype against biotrophic stress when individually mutated (Thireault *et al.,* 2015). *JAZ13-OX* lines showed decreased transcriptional induction of several key JA defense genes (*AOS/LOX3* and *MYC2*) in response to wounding (Thireault *et al.,* 2015). This attenuated defence response significantly increased the susceptibility of the *JAZ13-OX* line to *Spodoptera exigua* larvae feeding (Thireault *et al.,* 2015). Given this evidence it appears *JAZ13* is a negative regulator of the plant defence response against herbivory.

A single RNAi *JAZ10* silenced mutation was shown to increase susceptibility to *B. cinerea* colonization (Cerrundo *et al*., 2012). However, this phenotype was light wavelength dependent, under ambient lighting no difference was observed between the *jaz10* and Col-0 susceptibility levels, conversely under low red:far red light conditions *jaz10* displayed significantly increased susceptibility to *B. cinerea* (Cerrundo *et al*., 2012). However, a *jaz10* knockout line displayed increased susceptibility to *B. cinerea* when compared to Col-0 in this chapter, independent of light or dark light conditions (Fig.4.2). Furthermore, a single *JAZ10* RNAi silenced line and *JAZ10* knockout T-DNA insertion line both exhibited marginally increased chlorosis and increased symptom severity when challenged with *P. syringae* (Demianski *et al.,* 2012).

These phenotypes along with the result that *JAZ6* is a regulator of the defence response pathway suggest the level of functional redundancy among the JAZ proteins may not be as high as previously thought. If the single *jaz6* mutant and Col-0 were only inoculated at subjective dawn no difference would have been

observed between the lines. It could therefore be the case that research which have only screened single JAZ mutants under one condition and observed no phenotypic differences to WT may not have captured the correct condition for the JAZ to display its phenotype.

4.3.4 JAZ proteins can act as either positive or negative regulators of the plant biotic defence pathway

Susceptibility to the hemibiotrophic pathogens, *F. oxysporum* and *P. syringae*, has been shown to involve the JA signalling pathways. COI1 is essential for JA perception and activating the JA defence TFs, hence without it plants are unable to active their JA signalling cascade which is essential for defence against *B. cinerea*. However, defence against hemibiotrophic pathogens relies upon the SA signalling pathway, which can be counteracted by the antagonistic hormone, JA, it is therefore thought JA increases susceptibility to hemibiotrophic pathogens (Brooks *et al.,* 2005). This is confirmed by the high level of resistance *coi1* mutants have against both *F. oxysporum* (Thatcher *et al.,* 2009) and *P. syringae* (Kloek *et al.,* 2001). Whereas plants have significantly increased susceptibility to *B. cinerea* when *COI1* expression is reduced (Ferarri *et al.,* 2007).

Specific JAZ proteins are positive regulators of the defence response against *P. syringae* (JAZ5/10) (de Torres Zabala *et al.*, 2015) whereas others are negative regulators of the defence response against *P. syringae* (JAZ7) (Thatcher *et al.,* 2016). JAZ1 can act as both a positive and negative regulator of defence responses, with negative regulation of host *B. cinerea* defence observed, as well as positive regulation of the *P. syringae* defence response (Thines *et al.,* 2007). JAZ10 however, has been shown to be a positive regulator of defences against *B. cinerea* (Fig.4.2) and *P. syringae* (Demianski *et al.,* 2012). Also, when inoculated at night JAZ6 negatively regulates the defence responses against *B. cinerea* (Fig.4.2).

As outlined, it could be that when *JAZ6* is reduced in expression and resistance to *B. cinerea* in response to night inoculations is subsequently increased, the

protein assumed to be a positive regulator of the defence response previously bound by JAZ6 is then bound by another JAZ protein. The JAZ protein now regulating the defence response could be JAZ10, a positive regulator of defences against *B. cinerea*.

These complex phenotypes show that the JAZ proteins can act in many different ways in biotic stress and further work should be carried out to elucidate the role of each JAZ in plant-pathogen interactions. It could be the case that the antagonism that exists between the SA and JA defence pathways is fine-tuned by specific JAZ proteins, proteins such as JAZ1 which can both positively and negatively regulate the defence response pathways depending upon the pathogen lifestyle. However, this hypothesis needs further investigation. It would be especially interesting to find out exactly how each JAZ is mediating the defence response.

4.3.5 JAZ6 mediates the link between the circadian clock and the defence response by binding TFs involved in the clock and defence against *B. cinerea*

The JAZ6 protein links the circadian clock to the defence response against *B. cinerea.* How JAZ6 is mediating this response remains to be elucidated and as JAZ6 exhorts its repressive action at a protein level, it is difficult to link the previously obtained transcriptomic data to the JAZ proteins. Therefore, a technique to elucidate the interactions of the JAZ6 protein was employed with the hope of finding either proteins linking JAZ6 to the circadian clock or/and the *B. cinerea* defence response.

4.3.5.1 Y2H results require *in planta* validation

Protein to protein interactions can be detected using numerous techniques, including pull-down-assays coupled with mass spectrometry (PA-MS) or immunoblots, Co-IPs, BiFC and Y2H (a more extensive list can be found in the review by Bruckner *et al.,* (2009)). Each technique comes with its own set of

advantageous and drawbacks. Y2H was employed in this study due to the ease, speed and inexpensive nature of the technique as well as the availability of the whole Arabidopsis TF collection (1956 of which were cloned and screened) (Pruneda-Paz *et al.,* 2014).

Moreover, unlike PA-MS, Y2H does not discriminate based on protein abundance (von Mering *et al*., 2002). JAZ6 has been shown to form larger hetero and homodimers with members of the JAZ family as well as other protein families (Chini *et al.,* 2009). Such protein complexes will consist of proteins with different ratios of abundance hence the bias PA-MS displays towards highly abundant proteins may increase the likelihood of false results by only detecting highly abundant proteins within these complexes.

Although fast, inexpensive and unbiased toward protein abundance, the reliability of Y2H results are generally low and require validation using at least one, preferably two, of the techniques outlined above. The results obtained in this chapter were an initial screen to give an indication of how JAZ6 might be linking these two pathways and further validation using *in-planta* techniques is required before any of these results can be confirmed. The reason extensive validation of results obtained via Y2H is required is due to the high rate of false positive and false negative results generally associated with this technique (Bruckner *et al.,* 2009).

False negatives within Y2H are protein-to-protein interactions that are not detected due to the limitations of the technique and the specifics of each screening method used. When screening for interactions between Arabidopsis proteins false negatives are often detected in Y2H as yeast lack the post-translational protein modification machinery found in Arabidopsis, hence proteins may show different interaction patterns. If the modification is known then it is possible to co-express the protein modification enzyme in yeast. Moreover, false negatives may arise due to the interactions being transient. These false negatives are the largest issue when dealing reproducibility with Y2H screens, two large-scale Y2H screens were performed following the same

methodology and only 30% of the positive interaction were shared between both screens, moreover only 12.5% of known interactions were detected in both screens (Ito *et al.*, 2001).

It is not only false negatives that reduce the reliability of Y2H screens, Y2H can also detect interactions which are not truly reflecting what is happening in the biological system from which the protein is derived, these are known as false positive interactions. False positives can arise due to a number of factors, commonly it is due to the increased expression of the bait and/or prey proteins in the yeast cells which does not reflect their true expression levels within their natural biological system. Also, as mentioned, proteins may not be in their correct conformation due to the lack of post-translational protein modification machinery in yeast cells, this incorrect conformation may allow them to bind to proteins which they may not when they are fully folded in their natural systems. Or, as previously outlined, it could be that the proteins have a structure which allows unspecific binding to many proteins and hence are "sticky", the concept of "sticky" proteins was outlined in Vidalain *et al.,* (2004). It could also be the case that although proteins are able to interact in Y2H and *in-planta* the proteins are not localised to the same cellular compartment so will never interact with one another.

Another drawback of using Y2H is it only detects direct protein-to-protein interactions. Using a technique such as PA-MS would allow the identification of full protein complexes that JAZ6 is involved in. This would be especially useful for JAZ6 as like most other JAZ proteins it is known to form larger protein complexes. For example, JAZ6 has been shown to be able to directly bind to Topless via the JAZ6 EAR Domains (Causier *et al.* (2012); Shyu *et al.* (2012)). Topless then recruits histone deacetylases form a repressive protein complex.

4.3.5.2 Reliability of this dataset

Without *in-planta* validation this dataset is a preliminary indication of how JAZ6 is mediating the link between the plant defence response to *B. cinerea* and the

circadian clock. The reliability of this each dataset used as an initial screen to identify unique JAZ6 interactors will be discussed.

Each interaction was repeated three times and unless an interaction repeated at least once in these three repeats it was deemed a false positive. The JAZ6: TF library screen yielded 25 repeatable interactions, of these 12 interactions were detected in only two of the three replicates. Moreover, four interactions in this database were detected only once and were dismissed as false positives and not included in the final 25. The mixed JAZ pool against the full TF library screen yielded 41 repeatable positive interactions, of these, 14 interactions were detected in only two of the three replicates. Seven interactions were also found in this screen to repeat only once and hence were dismissed as false positives. The JAZ5 screen against 768 selected TFs in the library was only repeated twice, four interactions repeated in both replicates whereas 16 only repeated once. The TF subset screen against individual JAZ (JAZ5/6/7/10) proteins also showed reproducibility problems; on average each JAZ protein bound 27 TFs in the TF subset list, with an average of 12 of these interactions were seen in only two of the three replicate screens. Furthermore, on average four interactions were excluded for each JAZ protein, as they did not replicate.

Approximately half of all interactions documented were only detectable in two of the three replicates for each screen. This low reproducibility raises questions to the reliability of each dataset and encourages caution in interpretation of the data. The low reproducibility between repeats could be due to the nature of Y2H yielding high positive/negatives.

To further check the reproducibility of this screen comparisons between datasets were made (Table 4.3). The results obtained from screening the full TF library against either the JAZ6 protein or the mixed JAZ pool was compared with the results obtained screening the TF subset list only against individual JAZ proteins. It was found there was a high level of reproducibility between screens, 41 (82%) of the results obtained match the previous mixed JAZ and JAZ6 results. However, eight proteins (16%) show different results in this

individual JAZ5/6/7/10 screen compared to the mixed JAZ pool and JAZ6 screens. This can partially be attributed to the pooling of JAZ proteins finding fewer interactions compared to when JAZ proteins are screened individually (Appendix 6) or the nature of the individual proteins as previously outlined.

Another concern with regards to the reliability of this dataset is the strength of interactions. For example, the pooled mixed JAZ screen against the TF library showed a total of 41 interactions, 70% (*n* = 29) of these exhibited a score of only one (see methods for scoring system) on SD-LTH plates and furthermore, only 22% (*n* = 9) of these interactions were exhibited on both selective medias (SD-LTH and SD-LTA). Three of the interactors detected on both selective medias were the known interactors of the JAZ proteins: MYC2/3/4 and JAM2, all of which scored a three on both selective medias. No other interactions scored three on both medias, it could therefore be the case that other interactions are weaker between the JAZ proteins and the TFs, to determine the validity of these interactors individual protein to protein interactions will be further investigated and compared to previous results.

4.3.5.2.1 Interactors detected in this dataset are comparable to previous results

It was first ensured this screen was detecting known interactors of the JAZ proteins, MYCs2/3/4 (Fig.4.8), all JAZ proteins screened reproducibly interacted with all MYC proteins screened, as outlined this result concurs with previous research. Moreover, JAM2 has previously been shown to interact with all JAZ proteins apart from JAZ7 and JAZ12 in a Y2H assay performed in the same direction as the Y2H in this chapter (Song *et al.,* 2013). These researchers then confirmed the interactions between JAM2 and JAZ1 and JAZ10 *in-planta* using BiFC assays. Results obtained in this chapter when screening JAZ5, JAZ6 and JAZ10 against JAM2 agree with the results by Song *et al.,* (2013), however in Table 4.3 an interaction was observed between JAZ7 and JAM2. This interaction was not observed on the SD-LTA media, only the SD-LTH, and so could be a false positive, as is expected in Y2H, or it could be due to the

differences in the screens, Song *et al.,* (2013) used different vectors to those used in this study and a beta-galactosidase reporter gene system which may be more stringent than the *HIS3* reporter gene.

This screen also showed NIG1 is able to bind with JAZ5 and JAZ10 in Y2H (Table 4.3). Previous results have showed NIG1 to bind to JAZ1/2/5/6/8/10/11 in Y2H and the interactions between NIG1 and JAZ1/10 were confirmed *in-vivo* (Qi *et al.,* 2015). Three of the four interactions in the screen in Table 4.3 agree with the results obtained by Qi *et al.,* (2015), with the only variation being JAZ6 was not found to bind to NIG1 in Appendix 7, however Qi *et al.,* (2015) observed an interaction between these two proteins, albeit a weak one. Again this could be due to differences in the Y2H assay, Qi *et al.,* (2015) employed the same vector and reporter gene system as Song *et al.,* (2013) mentioned previously.

In Table 4.4 it can be seen EIN3 interacts with JAZ6 and did not interact with JAZ5/7/10 in the individual JAZ screen nor did EIN3 interact with the JAZs when pooled (Mixed JAZs). EIN3 has previously been shown to interact with JAZ1/3/9 in a Y2H screen and then this interaction was confirmed *in-planta* using BiFC and Co-IP assays (Zhu *et al.,* 2011). The JAZ pool used for the mixed JAZ screen contained JAZ1 and JAZ3 so EIN3 ought to have interacted with the pool of mixed JAZs. However, as previously mentioned, by pooling the JAZ proteins several interactions were missed (Appendix 6) so it is likely in single JAZ1:EIN3 or JAZ3:EIN3 or JAZ9:EIN3 screen interactions would be observed.

4.3.5.3 JAZ proteins interact with a wide range of TFs

4.3.5.3.1 Interactions were found between multiple JAZ proteins and the TF library

The TFs outlined in this section are not exclusive interactors of JAZ6, they either bound many other JAZ proteins as well as JAZ6 or only bound to either JAZ5, JAZ7 or/and JAZ10 so based on this screen alone, it is unlikely these are directly involved in the differential resistance observed to *B. cinerea* depending on time

of day of inoculation. However, as outlined individual JAZ proteins show different roles in resistance against specific pathogens so it is still worth investigating these TF interactions as they may provide insight into how the JAZ proteins are fine-tuning the defence pathway.

4.3.5.3.1.1 JAM2 interacts with many JAZ proteins

JA-ASSOCIATED MYC2-LIKE 2 (JAM2) is expressed in adult leaves (Appendix 7) and functions alongside JAM1 and JAM3 as a negative regulator of the JA signalling pathway (Sasaki-Sekimoto *et al.,* 2013). JAM2 interacts with JAZ5, JAZ6 and JAZ10 (Appendix 7). This TF has been shown to share many targets with MYC2 (Including ERF1), only rather than activating their transcription, as MYC2 does, JAM2 acts antagonistically to MYC2 and represses TF expression (Sasaki-Sekimoto *et al.,* 2013). JAZ proteins bind both MYC2 and JAM2 (Appendix 7) (Song *et al.,* 2013); it could be that JAZ proteins mediate the JA response pathways by fine-tuning the expression of both these TFs JAZ proteins. However, further work investigating *in-planta* interactions between JAM2 and the JAZ proteins under different conditions is needed to support this speculation is needed.

4.3.5.3.1.2 FRS3 interacts with many JAZ proteins

FAR1-RELATED SEQUENCE 3 (FRS3) is expressed in adult leaves and is one of 14 genes in the FAR1-related sequences family (Arabidopsis Genome Initiative, 2000), which have been shown to play vital roles in Arabidopsis far red light responses (Lin and Wang, 2004). This study revealed FRS3 is capable of binding to JAZ5/6/7 in Y2H (Table 4.3), FRS3 has never been shown to interact with these three JAZs before but it has been documented to bind to JAZ3 in Y2H (Arabidopsis Interactome Mapping Consortium, 2011).

The JAZ proteins interacting with FRS3 could be the link between the far-red response pathway and the JA signalling pathway. Previous research has linked far-red ratios to the JA signalling pathway and *B. cinerea* susceptibility

(Cerrundo *et al.*, (2012); Leone *et al*., (2014)). Low red: far-red (R:FR) ratios reduce JA signalling and JA related defence responses and hence increase susceptibility to *B. cinerea*, this reduction in JA signalling has been shown to be modulated by JAZ10 (Cerrundo *et al.*, 2012). DELLA proteins mediate the modulation of JAZ10 under different light wavelengths (Leone *et al*., 2014). Other JAZ proteins could also linking these pathways, however, this has yet to be tested. It could be postulated that FRS3 is modulating the JAZ and JA repression responses in times of low R:FR ratios, by directly interacting with JAZ proteins. It could even be the case that FRS3 plays an indirect role in the negative regulation JAZ10 has on the host defence responses against *B. cinerea* (Cerrundo *et al.*, 2012) and *P. syringae* (Demianski *et al.,* 2012), however, again this is entirely speculative and would need to be validated by experimental work *in-planta*.

4.3.5.3.1.3 NIG1 binds with JAZ5 and JAZ10

NACL-INDUCIBLE GENE 1 (NIG1) is expressed in adult leaves (Appendix 7) and has been is targeted by the JAZ family for repression (Qi *et al.,* 2015). This screen showed NIG1 is able to bind with JAZ5 and JAZ10 in Y2H (Table 4.3). NIG1 has been named MYC5 due to the discovery that NIG1 acts redundantly with MYC2/3/4 in stamen development and the production of seeds (Figueroa *et al.,* (2015); Qi *et al.,* (2015)). However, unlike the other three MYC proteins (2/3/4) NIG1 has not been shown to interact with other MYCs (Appendix 7) and the role NIG1 plays in pathogen defence has yet to be investigated, therefore it cannot be speculated how this interaction may play a role in pathogen defences. It is likely at this point that this interaction is based on regulating JA responses during stamen development.

4.3.5.3.1.4 TCP21 interacts JAZ5, JAZ6 and JAZ10

TCP DOMAIN PROTEIN 21 (TCP21), also named CCA1 HIKING EXPEDITION, (CHE), is expressed in adult leaves and is a regulator of the circadian clock (Appendix 7) (Pruneda-Paz *et al.,* 2009). TCP21 has never previously been

shown to interact with any of the JAZ family or the MYC2, 3 or 4 (Appendix 7). In Table 4.3 it can be seen TCP21 interacted with JAZ5/6/10.

TCP21 has strong links to the central oscillator; it is rhythmically regulated by CCA1 and LHY (Pruneda-Paz *et al.,* 2009). Moreover, TOC1 cannot directly bind to DNA so TCP21 binds to the CCA1 promoter region allowing TOC1 to repress CCA1 expression (Pruneda-Paz *et al.,* 2009). This protein also has links to the immune response, TCP21 contributes to the rhythmicity in the SA levels and upregualtes host SA production upon detecting a biotrophic pathogen by positively regulating the key SA biosynthetic enzyme, isochorismate synthase 1 (ICS1) (Zheng *et al.,* 2015).

The interaction between TCP21 and JAZ5 and JAZ10 may contribute to the *jaz5/10* mutant's compromised immune response to *P. syringae* (de Torres Zabala *et al.*, 2015) given TCP21's role in the SA biosynthetic pathway (Zheng *et al.,* 2015). This gene may also contribute to the rhythmicity observed in the defences against *P. syringae* in wild-type lines (Bhardwaj *et al.* 2011).

As well as these links to the clock and plant immunity against a hemi-biotrophic pathogen, the expression levels of the *TCP21* transcript were shown to be DE in response to *B. cinerea* inoculation at dawn compared to night (see Chapter.3). *TCP21* was is in the DOWNDOWN18 grouping showing it to be significantly more downregulated in response to inoculated at dawn compared to night (Table 3.5). It was postulated *TCP21* is significantly more repressed in response to dawn compared to night inoculations as *TCP21* is a positive regulator of SA biosynthesis, hence repressing this gene will reduce SA biosynthesis in response to dawn inoculations. Due to the antagonism that exists between the SA and JA pathways, repressing SA biosynthesis will reduce the repression SA has on the JA pathways. In turn, JA pathways will therefore more responsive to inoculations at dawn compared to night.

This theory is very speculative at this point, however, if this is the reason for the greater repression of *TCP21* in response to dawn compared to night

inoculations then the JAZ proteins could be involved in this. It could be that *TCP21* acts in the SA/JA antagonism and acts in a complex with JAZs to repress JA related genes while TCP21 also activates SA related genes. The plant therefore represses the expression of this transcript to prevent the repressive action on the JA pathway when defending against a necrotrophic pathogen, and the plant represses *TCP21* expression more in response to dawn inoculations compared to night inoculations and this contributes to the time-of-day dependent immunity.

Given that *JAZ6* is the only JAZ protein found to disrupt the circadian defence pattern against *B. cinerea* it is more likely that proteins, which exclusively bind to JAZ6, play a stronger role in the circadian defence pattern than those that are bound by JAZ6 as well as JAZ proteins, which have no influence on this circadian defence pattern (such as JAZ5 and JAZ10) (Fig.4.2).

4.3.5.3.2 JAZ6 interacts with TFs involved in a wide range of biological processes

Perhaps the most interesting proteins in this screen are the seven proteins (MYB_LIKE, AT4G23860, WOX12, NAC010, ZF1, EIN3 and FHY3) that were not bound by the mixed pool of JAZs or JAZ5/7/10 and were only found to interact with JAZ6 (Table 4.4). Any of these interactions could be key linking the circadian clock and JA defence pathway against *B. cinerea*. These candidates will be further investigated to gauge the likelihood of them acting as links between the clock and the host defence response.

4.3.5.3.2.1 JAZ6 interacts with TFs involved in abiotic stress tolerance and development

WUSCHEL-related homeobox 12 (WOX12) is a member of the WOX protein family, the main function of this family has been characterised as embryonic pattern formation in early zygotes, flower development and root organogenesis (Constanzo *et al.,* (2014); Haecker *et al.,* (2004); Liu *et al.,* (2014)). JA is known

to fine tune the plant balance between defence and growth, mainly by inducing defence responses and repressing root growth (Havko *et al.,* 2016). So it is possible that JAZ6 binding to WOX12 is a mechanism within this fine tuning response.

Zinc finger protein 1 (ZF1) is a transcriptional repressor that plays a vital role in abiotic stress tolerance by repressing plant growth (Kodaira *et al.,* (2011); Sakamoto *et al.,* (2004)). ZF1 is expressed in adult leaves and has only been shown to bind to JAZ6 in this screen and has never been documented to interact with any other members of the JAZ family (Table.4). As well as being heavily involved in abiotic (high salinity and low temperature) stress responses, ZF1 has several links to the defence response. *ZF1* expression has previously been shown to increase significantly in response to chitin (Libault *et al.,* 2007) and ZF1 possesses a conserved EAR domain (Ciftci-Yilmaz *et al.,* 2008). Repressors with this domain have been largely implicated in the biotic stress response, for example ERF4 contains a conserved ear domain through which it represses the expression of *PDF1.2* by binding to its promoter region (McGrath *et al.,* (2005); Yang *et al.,* (2005)).

It could be that ZF1 represses plant growth under both abiotic and biotic stress conditions. JAZ6 directly binding ZF1 allows the repression of plant growth in response to JA-Ile that is released upon the perception of a necrotrophic pathogen. Why JAZ6 would be the only JAZ protein to regulate this process is as yet unknown and further investigation is required to elucidate this.

4.3.5.3.2.3 JAZ6 directly interacts with a key regulator of the host defence network against *B. cinerea*

EIN3 has previously been shown to be a positive regulator in the defence response against *B. cinerea*, with *ein3-1* mutants showing significantly enhanced susceptibility to *B. cinerea* (infection time not stated) (Zhu *et al.,* 2011). EIN3 positively regulates the host defences against necrotrophic pathogens by binding to the *ERF1* promoter region and inducing *ERF1* transcription (Solano *et al.,* 1998). ERF1 then binds to the GCC-Box motif upstream of genes essential

to host defence against *B. cinerea* (such as *PDF1.2, b-chitinase* etc.) and induces transcription (Solano *et al.,* 1998). EIN3 is thought to be a master regulator in this defence response, so much so that the interaction between EIN3 and MYC2 is hypothesized to be the 'regulatory switch' between activating the wounding and herbivore response pathways (positively regulated by MYC2) and the necrotrophic defences and root hair development pathways (positively regulated by EIN3) (Song *et al.,* (2014); Zhu *et al.,* (2011)). JAZ6 binding to and regulating such a crucial TF would explain the powerful effects losing *JAZ6* expression has on defence against *B. cinerea*.

As mentioned, JAZ1, JAZ3 and JAZ9 have shown to bind to EIN3 (Zhu *et al.,* 2011). Unfortunately, no mutants with compromised expression of *JAZ1, JAZ3* or *JAZ9* were screened for differences in resistance to *B. cinerea* depending on the time of day so it is difficult to determine if JAZ6 is the only JAZ linking the defence pathway to the circadian clock or if other JAZ proteins could also link the two pathways. Based on the expression profiles of *JAZ3* and *JAZ9* compared to *JAZ6* (Fig.4.16) *JAZ9* does not show a strong diurnal transcriptional pattern hence this protein is unlikely to be under the same circadian regulation as *JAZ6.* Moreover, *JAZ1* did not show a significant circadian expression pattern hence is not shown in Fig.4.16. *JAZ3*, however, (orange line in Fig. 4.16) shows a highly rhythmic expression pattern, with a peak at a similar time to *JAZ6* (green line in Fig.4.16), it could therefore be speculated that a *jaz3* mutant may also disrupt the circadian defence pattern observed against *B. cinerea*. However this is entirely speculation at this point and mutants of all JAZ genes should be screened for differences in resistance to *B. cinerea* depending on time of inoculation to see if any other JAZ proteins are linking the defence and the circadian pathways.

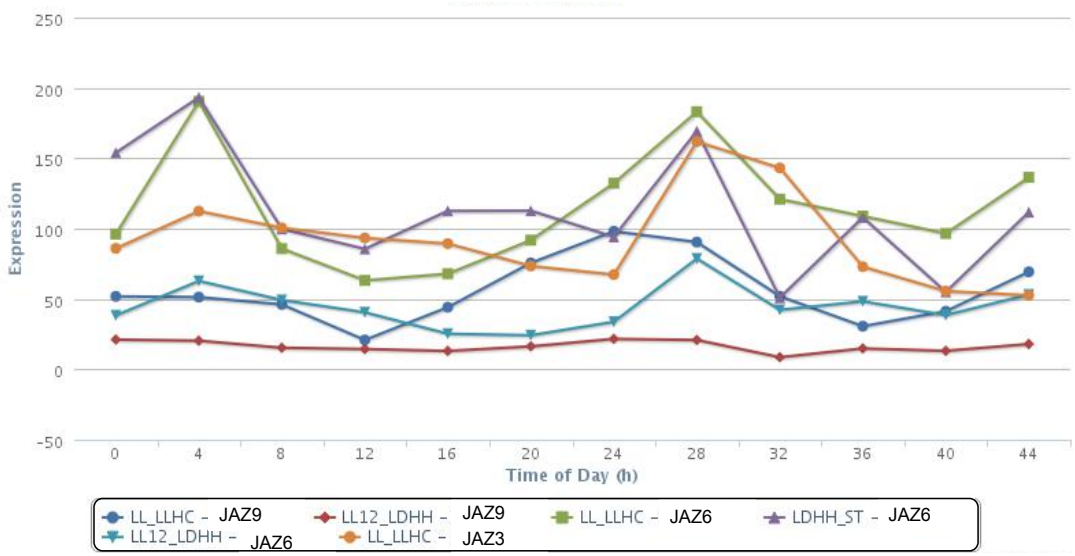


Figure 4.16 – *JAZ3/6/9* display a circadian expression pattern under numerous growth conditions. LL_LLHC Seedlings grown on agar for 9-days under CL and cyclic hot:cold conditions. Seedlings were then placed in LL and constant temperature for 24 h and then harvested. LDHH_ST Plants were grown on soil under 12:12 LD conditions and kept at a constant temperature for 35 days - leaves were then harvested. LL12_LDHH Seedlings were grown on agar for 7-days under 12:12 LD conditions and kept at a constant temperature. Seedlings were then placed in LL and constant temperature for 24 h and then harvested. All experiments were carried out on Col-0 plants under a constant temperature of 22°C. Expression – RNA expression levels (log^2) (Blasing *et al.*, 2005; Covington *et al.,* 2008). Image from Diurnal (Mockler *et al.,* 2007).

To determine if JAZ6 was modulating the circadian defence pathway via EIN3 repression, an *ein3-1* mutant with reduced *EIN3* expression was inoculated at subjective dawn (ZT 0) and subjective night (ZT 18) with *B. cinerea* spores and lesions were measured 72hpi (Fig4.13). As previously outlined *ein3-1* has previously been shown to be more susceptible to *B. cinerea* inoculation compared to Col-0 (infection time not stated) (Zhu *et al.,* 2011). However, in Figure 4.13 it can be seen *ein3-1* was found to be equally as susceptible as Col-0 when inoculated at subjective dawn and more resistant to *B. cinerea* compared to Col-0 when inoculated at subjective night under LD conditions. Appendix 7 is the result of one experimental replicate so it is difficult to say if this is truly reliable and repeats would be required to confirm this.

The differences in results seen in Figure 4.13 and Zhu *et al.,* (2011) are likely due to lack of experimental replicates used for Figure 4.13 but could also be

attributed to differences in experimental conditions. For example, variations between light wavelengths have previously been shown to influence *B. cinerea* infection outcomes (Chico *et al.,* 2014) and as have differences in fungal incubation conditions. Hevia *et al.,* (2015) showed *B. cinerea* B05.10 can exhibit differences in virulence due to 12:12 LD entrainment during incubation which was employed by Zhu *et al.,* (2011), however studies carried out in this chapter all *B. cinerea* was grown under DD.

If data in Figure 4.13 is accurate, it appears a mutant with reduced *EIN3* expression (*ein3-1*) shows no difference when inoculated at subjective dawn compared to subjective night with *B. cinerea* (Fig. 4.13). Given this, a hypothesis that JAZ6 (and potentially other JAZ proteins) is exerting its influence on the host *B. cinerea* defence pathway by directly repressing *EIN3* more in response to inoculations at night than those at dawn was formed. This would explain not only the increased resistance to *B. cinerea* in response to inoculations at dawn compared to night, but also the increased *PDF1.2* expression in response to dawn inoculations compared to night observed by Ingle *et al*., (2015).

When *JAZ6* expression is reduced in the plant and the time of day dependent differences in resistance are then lost, several molecular events could be occurring at the EIN3 level (Fig.4.17). Scenario one (Fig.4.17 – A) in Col-0 is that EIN3 is free to activate downstream necrotrophic defences and not repressed by JAZ6 or any other JAZ in response to inoculations at dawn however in response to inoculations at night, EIN3 is bound and unable to activate downstream defences (Fig.4.17 – A). Scenario two is a little more complex (Fig.4.17 – B), due to the high level of redundancy observed in the JAZ family; it could be that in the absence of JAZ6 in response to inoculations at dawn another JAZ protein binds EIN3. This JAZ protein may not repress EIN3 as strongly as JAZ6 and may therefore allow EIN3 to activate the defence pathway.

In the absence of JAZ6 (in a *jaz6* mutant) the EIN3 protein would therefore be either completely unrepressed in response to dawn or night inoculations (Fig.4.17 – C) allowing for constitutive expression of the defence response

independent of time of inoculation. EIN3 could be bound by other JAZ proteins in response to both dawn and night inoculations (Fig.4.17 – D) if scenario two was accurate. This JAZ protein may not have a strong diurnal expression pattern (e.g. Fig.4.17– JAZ1/9) and would provide constitutive repression of EIN3 in response to inoculations both at dawn and night. The repression with this JAZ protein may not be as strong as the interaction between JAZ6 and EIN3 hence EIN3 expression and downstream defences would still be activated (Fig.4.17– D).

Constitutive overexpression of *JAZ6* (*JAZ6-OX*) conferred equal high levels of resistance to *B. cinerea* when inoculations occurred at dawn or night. Several mechanisms could confer this phenotype with relation to JAZ6 interacting with EIN3. It could be that when inoculated at dawn or night the EIN3 to JAZ6 interaction is the same and no EIN3 protein is available to activate downstream defences. EIN3-like (EIL) TFs are functionally redundant and highly homologous to EIN3, with single mutants of either often conferring no differential phenotypes (Boutrot *et al.,* 2010). It is therefore possible in the absence of EIN3 activity; EIL TFs resume the role EIN3 once played in activating the defence pathway (Fig.4.17 – E). It is also possible that the JAZ proteins previously shown to weakly bind EIN3 could also be binding EIL1 in response to dawn and night inoculations, however as was previously the case it maybe that these interactions are weaker than what is observed with JAZ6 hence defence is still activated (Fig.4.17 – F).

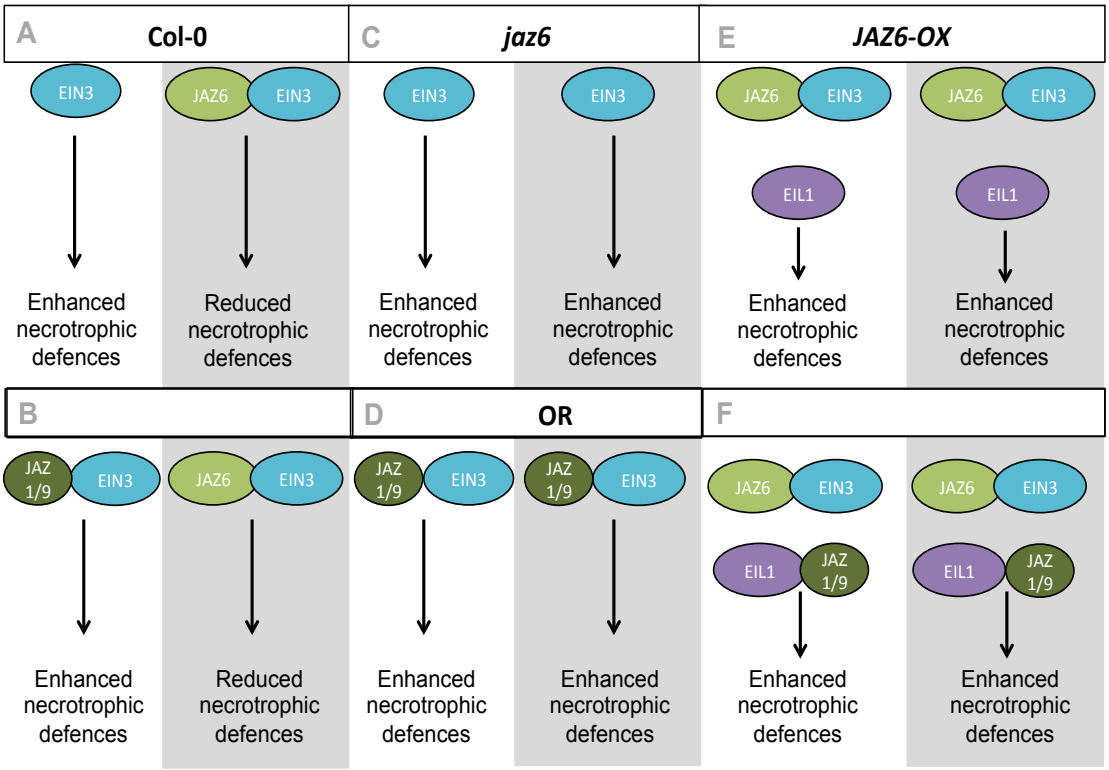


Figure 4.17 – EIN3 regulation of defence in the Col-0, *jaz6* and *JAZ6-OX* lines after dawn and night inoculations. Col-0 plants inoculated with *B. cinerea* at night show increased susceptibility compared to those inoculated at dawn. In response to dawn inoculations it is predicted EIN3 activates the necrotrophic defence pathway. EIN3 and JAZ6 may interact in response to night inoculations; this interaction could repress EIN3's positive defence regulation and increase Col-0 susceptibility (A). Or it could be the scenario that EIN3 is repressed at dawn but its repression is only weak so defences are still activated, this is possibly mediated by another JAZ protein (B). In a *jaz6* mutant constitutively high resistance to *B. cinerea* may be mediated by the now unrepressed EIN3 (C). Or it may be that EIN3 is repressed by another JAZ non-diurnal JAZ protein such as JAZ1/9 (D). This JAZ protein may not repress EIN3 as strongly as JAZ6 did hence activation of defence still occurs. In a JAZ6-OX line where constitutively high levels of resistance was also observed, JAZ6 could be binding and repressing EIN3 at all times of day. The absence of EIN3 may cause a redundant EIN3 TF such as EIL to activate the defence response (E). It is possible EIL1 does not go unrepressed and that constitutive JAZ proteins such as JAZ1/9 weakly and constitutively repress EIL1 (F).

4.3.6 Interactors of JAZ6 need to be confirmed *in-planta*

This Y2H screen has given a good indication of how JAZ6 could be regulating the defence pathway against *B. cinerea* differently in response to inoculations at dawn compared to at night. However, this screen has only shown what JAZ6

could be binding and not what it is binding *in-planta*. From here it is crucial the binding partners of JAZ6 are confirmed *in-planta*, ideally a transgenic line expressing *JAZ6* under its native promoter with a tag would be generated. This line would allow for PA-MS to be performed on plants both mock and infected at dawn and night, giving information on what JAZ6 is binding to differently after dawn compared to night inoculations. Moreover, this line would also bring down full protein complexes rather than just direct targets so the full regulatory complex JAZ6 is involved in could be elucidated.

If confirmed *in-planta* mutant of the binding targets of JAZ6 could then be screened for differential immunity in response to inoculation at different times of day (as performed for *EIN3*). Those genes that showed no difference in resistance to *B. cinerea* between dawn and night inoculations could then be considered regulators of the circadian defence response against *B. cinerea*.

4.4 Conclusions

This research identified *JAZ6* as a regulatory link between the circadian clock and the defence response against *B. cinerea*. JAZ6 was then screened against almost all TFs uncovered in Arabidopsis using Y2H to gain insight into the complex interactions between the clock and the defence response. JAZ6 was binding a key positive regulator of the host defence response against *B. cinerea*, EIN3. These two TFs interacting may be the key to the JAZ6-dependent differential regulation of the immune response against *B. cinerea.*

Chapter 5: Mapping active gene regulatory elements mediating plant defence

5.1 Introduction

It has recently been shown that susceptibility of Arabidopsis to *Botrytis cinerea* depends on the time of the day when inoculation takes place (Chapter 3) . This chapter is aimed at investigating the differential chromatin accessibility and transcription factor (TF) binding throughout the Arabidopsis genome at two times of day when differential immunity to *B. cinerea* was observed. This would determine how the regulation of the defence response is differentially 'primed' to respond differently to inoculations at dawn or night.

Assay for Transposase-Accessible Chromatin coupled with high throughput sequencing (ATAC-Seq) was employed to examine differential defence priming throughout the genome at two times of day. Genome-wide mapping of chromatin accessibility using ATAC-Seq can identify areas of open chromatin as well as the interplay between chromatin and transcription factors (TFs). The recent outputs from The ENCODE Project Consortium (2004) have shown the value of a high throughput genome-wide examination of genetic elements.

When compared with other chromatin mapping techniques such as DNase I hypersensitive sites sequencing (DNase-seq) and Formaldehyde-Assisted Isolation of Regulatory Elements sequencing (FAIRE-seq) ATAC-Seq has several advantages. These include a shorter laboratory protocol (meaning transient interactions between DNA and proteins are more likely to be captured) and a reduced cell number is required (an extensive review of the advantages was carried out by Tsompana and Buck, 2014). Despite the numerous advantages, ATAC-Seq data has yet to be published on the Arabidopsis genome and up until August 2016 no data was available for ATAC-Seq on any plant species.

This technique makes use of a hyper-activated Tn5 transposase which both fragments and tags areas of open chromatin (tagmentation); these tagged areas

are then amplified and then undergo paired-end sequencing (Fig.5.1). The transposase can only insert itself and fragment areas of open chromatin hence why peaks of short reads are seen in regions of open chromatin (Fig..51). Moreover, this fragmentation can reveal chromatin occupied by TFs as these regions have a distinctive fragmentation footprint.

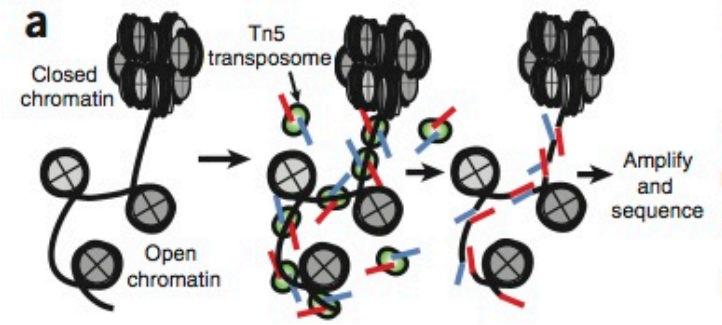


Figure 5.1 – ATAC-Seq technique principle. Grey crossed circles represent nucleosomes. Areas between nucleosomes are preferentially fragmented by the Tn5 transposase (small grey circle). Upon fragmenting the Tn5 also inserts adapter sequences (seen in red and blue) into DNA so reads can be easily amplified (Reproduced with permission from Buenrostro *et al*., 2013)

The data obtained from ATAC-Seq is of similar sensitivity and accuracy to that of DNase-seq (Tsompana and Buck, 2014). As such, it can be analysed using a comparable pipeline involving: de-multiplexing, trimming, aligning and filtering of reads. Areas of open chromatin can then be visualized using peak finding algorithms and genome viewers, areas of TF binding can also be identified using footprinting algorithms that allow Tn5 cuts to be analysed at a single base pair (bp) resolution. Regions bound by TFs can then be analysed for TF binding motifs and from this the likely TF linking the motif can be inferred.

This data can therefore reveal the differences in TF binding and chromatin accessibility at two times of day when differential resistance to *B. cinerea* has been observed. This data along with the transcriptomic profiling in Chapter 3 and the host defence network model inferred from a 48 hour (h) *B. cinerea* time-series experiment (Windram *et al.,* 2012) can pinpoint crucial network modules, and important regulatory sites in the genome, for effective plant disease resistance against *B. cinerea*.

5.1.1 Aims and objectives

The primary aim of this chapter was to identify genome-wide regions of active chromatin, and sites bound by TFs within open regions and how these sites change at different times of day. This can indicate what transcriptional changes are occurring to alter the host defence response to *B. cinerea* at different times of day.

Specific objectives:

1. Generate genome-wide ATAC-Seq data from Arabidopsis leaves harvested at two different times of day (Zeitgeber (ZT) 4 and ZT 10). Times indicated to be when plants are likely exhibiting different levels of resistance to *B. cinerea*.
2. Compare *cis*-regulatory regions between samples harvested at different times of day to identify changes over time and focus on those changes specifically involved in defence.

5.2 Methods

Previously it was seen that maximal resistance to *B. cinerea* occurred when plants were inoculated at Zeitgeber (ZT) 0 (subjective morning) and plants exhibited greatest susceptibility when inoculated at ZT 18 (subjective night) . *B. cinerea* takes between 10-12 h to germinate and influence the transcriptional defences in the host (Windram *et al.,* 2012). The times plants are therefore likely exhibiting different levels of immunity are 10 h after the above times, so ZT 10 (ZT 0 + 10 h) and ZT 4 (ZT 18 + 10 h). As such, three replicate samples were harvested at both ZT 4 and ZT 10. For each sample 2 g of fresh 4-week-old Columbia (Col-0) leaf tissue was harvested. Full methods can be found in Chapter 2. In brief, fresh tissue was ground with nuclear isolation buffer and nuclei were isolated and treated with Tn5 transposase. Following this, DNA was cleaned up and PCR was used to index DNA fragments. The indexed samples were subject to second clean up, DNA quantified and fragment lengths analysed. Two (ZT4.2, ZT4.3, ZT10.2 and ZT10.3) of the three replicate samples for each time point were then diluted and sequenced using paired end sequencing (PE). Once returned from sequencing several computational analysis steps were performed (Fig 5.2).

5.2.1 Computational analysis

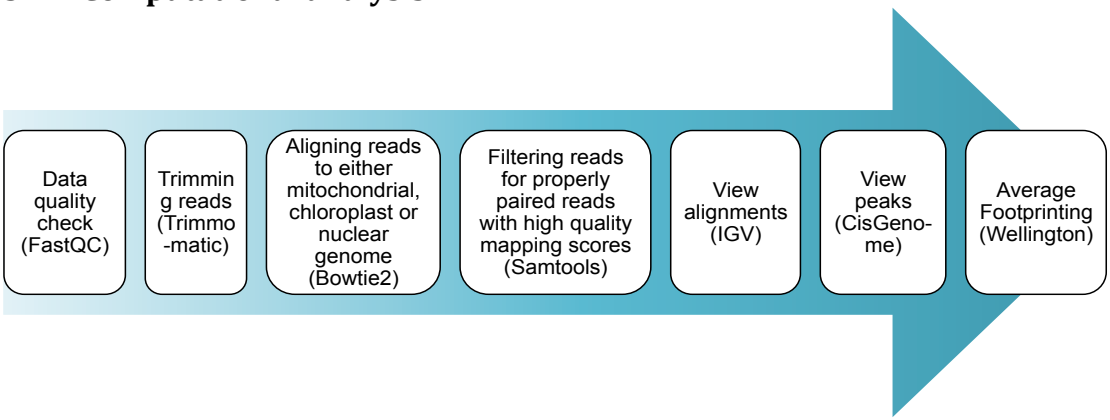


Figure 5.2 – Workflow for ATAC-Seq computational analysis. Data was first checked for sequencing quality and then adapters and low quality bases were trimmed. Trimmed reads were aligned to the Arabidopsis nuclear, mitochondrial or chloroplast genomes. Aligned reads were then further filtered to keep only high quality paired and well mapped reads. These alignments were viewed in IGV and peaks in reads mapping exclusively to the nuclear genome were viewed in CisGenome. Average binding profiles in protein binding motif regions were then investigated.

5.2.1.1 Quality control

Both prior to and after trimming sequencing data was quality checked using FastQC (Andrews, 2010) version (v) 0.11.4.

5.2.1.2 Trimming reads

Trimming was performed by Katherine Woolley-Allen (University of Warwick). Trimmomatic v0.33 (Bolger *et al.,* 2014) was employed to trim reads using the command:

java -jar path/Trimmomatic-0.33/trimmomatic-0.33.jar PE -threads 12 reads_path/A_1.fastq.gz reads_path/A_2.fastq.gz trimmed_files/A_1_tr.fastq trimmed_files/A_1_tr_unpaired.fastq trimmed_files/A_2_tr.fastq trimmed_files/A_2_tr_unpaired.fastq ILLUMINACLIP:NexteraPE-PE.fa:2:30:10:1:true TRAILING:3 SLIDINGWINDOW:4:15

This command directs the computer to use Trimmomatic v0.33 with settings for PE data using 12 threads. Input files for Trimmomatic are then specified (reads_path/A_1.fastq.gz reads_path/A_2.fastq.gz) along with the locations to store the newly trimmed files (trimmed_files/A_1_tr.fastq trimmed_files/A_1_tr_unpaired.fastq trimmed_files/A_2_tr.fastq trimmed_files/A_2_tr_unpaired.fastq). An adapter file is also required as an input (ILLUMINACLIP:NexteraPE-PE.fa:2:30:10:1:true) which contains the adapter sequences used in the Nextera protocol and allows Trimmomatic to identify and trim adapter sequences. A few parameters for Trimmomatic are then specified, SLIDINGWINDOW:4:15 directs the program to scan four bp at a time and if the average quality score per bp over the four is below 15 then the remainder of the read will be trimmed. TRAILING:3 specifies that once the sliding window command has reached the end of a read and there are three or fewer bp in the read remaining then the trailing command will check the score of each bp individually and if the quality score falls below 15 then they will be trimmed, along with the remainder of the read.

5.2.1.3 Aligning reads to the genome and filtering for high quality truly paired reads

Alignment was performed by Katherine Woolley-Allen (University of Warwick). Bowtie2 (Langmead and Salzberg, 2012) was employed to build index files for Arabidopsis TAIR9 genome. Many reads map to multiple organelles, to remove this ambiguity reads were mapped to each genome. The nuclear, mitochondrial and chloroplast genomes were constructed and indexed individually. Samples were then aligned to the mitochondrial and chloroplast index files, any reads that aligned to either organelle genome were filtered from downstream processing and moved to a separate folder. The remaining unmapped reads were then mapped to the nuclear genome. Parameters within software were set for PE reads.

'True pairs' were selected for, to be a true pair, two reads had to be located close to each other on the same chromosome and they had to be facing one

another. Sorted and indexed .bam files containing only 'true pairs' of high mapping quality (MAPQ) (>22) alignment scores were then created using Samtools. More specifically, the .sam file generated from the Bowtie2 aligner was converted to an indexed and sorted .bam file using Samtools. The following commands calculated information from only 'truly paired' reads within this .bam file by replacing the 'filter' command with the -f2 command in Samtools. MAPQ score distributions were generated for properly paired reads and subsequently plotted. This score indicates the likelihood of the read mapping uniquely, the higher the score the lower the probability of the reads belonging elsewhere. Insert size distribution for the 'true pairs' with a MAPQ score of above 22 were then calculated and subsequently plotted. A .bam file of truly mapped high quality reads (MAPQ >22) was generated, again using Samtools.

5.2.1.4 Reads mapping to the genome were viewed using IGV

The Arabidopsis TAIR9 genome was loaded into the Integrative Genomics Viewer (IGV) (Thorvaldsdóttir *et al.,* 2013) along with sorted, indexed and filtered .bam files of truly paired reads aligning uniquely to the genome. This gave insight into the frequency and distributions of aligned reads.

5.2.1.5 Peaks in reads were viewed using CisGenome

Files were converted from .bam into .bar files and viewed in the CisGenome browser (Ji *et al.,* 2008) along with the Arabidopsis TAIR9 genome annotation file to identify gene-associated peaks.

5.2.1.3 Average footprinting was performed using Wellington

Average footprinting was performed by Dr. Krzysztof Polanski (University of Warwick). Find Individual Motif Occurrences (FIMO) (Grant *et al.,* 2011) was used at a threshold of 10^{-4} to identify the coordinates of motifs within 300 bp upstream of transcriptional start sites (TSS) throughout the whole of the

Arabidopsis genome. These coordinates were translated into a .bed file, which then provided Wellington (Piper *et al.,* 2013) with locations to investigate the average footprinting pattern in each of the four samples.

5.3 Results

5.3.1 Sequencing data is of a high quality

5.3.1.1 Quality of read sequences is high

To gain an insight into the quality of the raw reads, a quality checker (FastQC) was used to analyze samples. Sample reads maintained a high level of quality for the full length of the reads across each individual base (1-75 bp) and this was consistent among all eight samples (a representative sample can be seen in Fig 5.3). Trimming was then performed as previously outlined and per base quality scores were unchanged. Given the high quality sequences the main purpose of trimming was to remove contamination from adapter sequences.

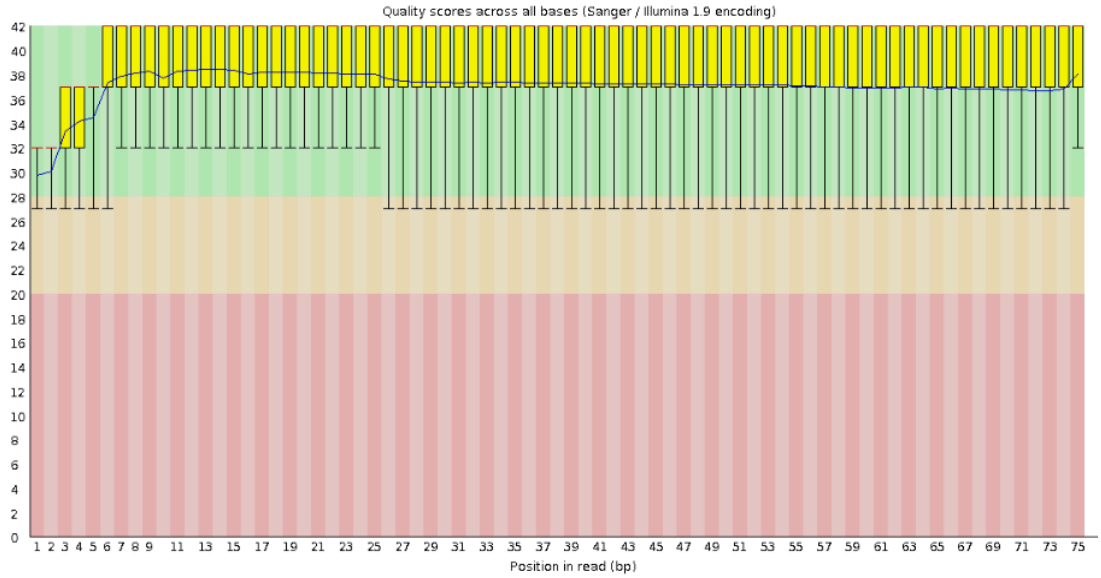


Figure 5.3 – FastQC quality checker showed sequencing was successful and of a high quality. Boxplot for the quality of each bp can be seen in yellow with a blue line displaying the mean per base quality. The y-axis shows per base quality score.. Anything in the red region are calls of a poor quality, those falling in the orange region are calls of a reasonable quality and those in the green region are calls of a good quality. Sample: ZT10.3 forward reads.

5.3.1.2 Filtering based on mapping score was vital to give high confidence data

MAPQ scores give an indication as to the uniqueness of a read-mapping event; so how probable it is that a particular read uniquely mapped to a specific region in the genome. MAPQ scores are generated from the aligner; Bowtie2 generates scores with a maximum quality of 42. These scores are based on probability; for example, if the probability of a read correctly mapping were 0.99 then the MAPQ score would be 20. As can be seen in Fig 5.4 the uniqueness of alignments was either of very high or very low quality hence filtering for higher scores was vital. Fig 5.4 is representative of alignments in all four samples to all compartment genomes (nuclear, mitochondria or chloroplast). Any reads that fell below a cut-off score of 22 were excluded from downstream analyses, as they were assumed unreliable. A threshold of 22 was arbitrarily selected based on the read score distribution, reads mapping below six were likely non-uniquely mapped and those that scored 42 were likely to be uniquely mapped, hence a score approximately between these two values was selected. Indeed, this could be slightly altered and would likely have a minimal impact on downstream analyses.

Low MAPQ scores can be due to many factors including mismatches between the reference genome and read due to the sequencer calling an incorrect base, incorrect alignment or could even be due to a true biological difference between the read and the reference genome, such as the read or reference genome containing a single nucleotide polymorphism (SNP).

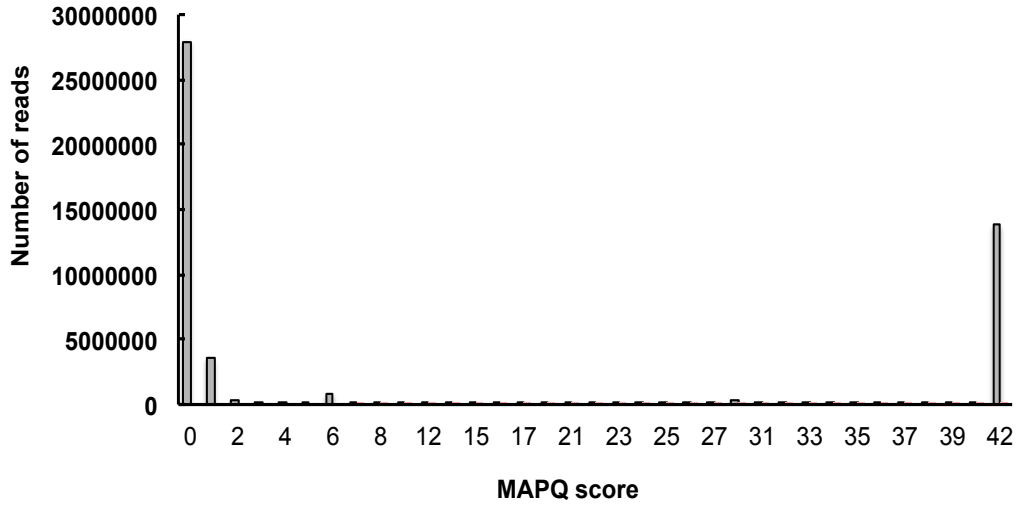


Figure 5.4 – Mapping scores of alignment quality showed many reads were non-uniquely mapped prior to filtering. MAPQ scores of reads from sample ZT4.2 aligning to the Arabidopsis nuclear genome. Reads were filtered for MAPQ scores above 22 to ensure reads were likely to be correctly mapped.

5.3.2 The majority of reads align to the chloroplast genome

Once it was established that reads were of a high quality, samples were first aligned to the mitochondrial and chloroplast genomes. Reads that aligned to either or both of these compartments, were filtered and the remaining reads were aligned to the nuclear genome. The proportion of reads aligning to each of the three genomes was calculated (Fig 5.5). Approximately 10% of reads aligned to the nuclear genome, approximately 10% aligned to the mitochondrial genome and the remaining 80% aligned to the chloroplast genome (Fig 5.5).

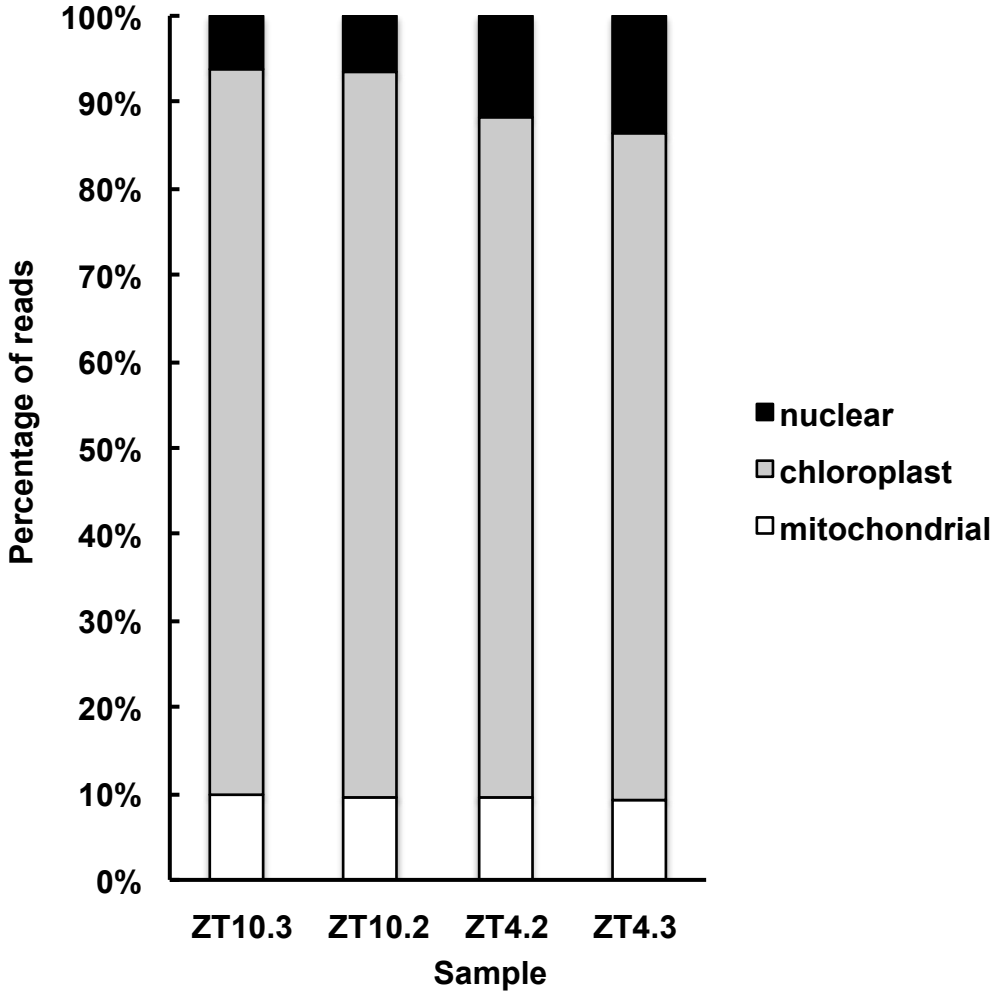


Figure 5.5 – The proportion of paired reads aligning to each organelle genome. Percentage of properly paired reads in each sample aligning to the chloroplast, mitochondrial or nuclear TAIR9 Arabidopsis genome with a MAPQ alignment score of above 22. Read numbers between sample varied, however, the average number of truly paired reads per samples with a MAPQ score of above 22 was ~70 million.

ATAC-Seq employs a Tn5 transposase which has a clear bias for cutting mitochondrial DNA, with the proportion of reads aligning to the mitochondrial genome varying between 10-50% in previous reports (Buenrostro *et al.,* 2015). The results in Fig.5.5 are in agreement with this finding, with 10% of paired reads aligning to the mitochondrial compartment. However, what is unprecedented is the high proportion of reads aligning to the chloroplast compartment. The ratio of plastid to nuclear DNA varies between 0.4%-20%, with the highest percentage observed in mature leaves (Rauwolf *et al.,* 2010). So even without nuclear enrichment the percentage of reads mapping to the

chloroplast genome should not exceed 20%. Nevertheless it appears Tn5 preferentially cuts the chloroplast genome in Arabidopsis (Fig.5.5). Since the analysis in Fig.5.5, data released by Wilkins *et al.,* (2016) also showed over 70% of reads aligned to the chloroplast in an ATAC-Seq experiment performed on *Oryza sativa.* This preference for the chloroplast is therefore not constrained to Arabidopsis and could be a universal problem for plant ATAC-Seq experiments.

5.3.3 Peak distribution along the genome does not reflect previous ATAC-Seq data

Although very few reads align exclusively to the to nuclear genome, it could still be possible that these nuclear reads are sufficient to detect peaks within the genome. CisGenome allowed the data to be visually mined for distinctive peak profiles that are evident when chromatin is accessible and unoccupied by nucleosomes (Fig.5.6). Peaks are the result of the accumulation of short reads in open regions due to the Tn5 or the DNase I enzyme preferentially cutting open regions of chromatin (indicated by blue arrows in Fig.5.6). When reads from both the F and R strands are aligned to the genome this will show two peaks of equal magnitude close to one another (Fig.5.6). This is due to a slightly increased number of positive strand starts compared to negative strand starts. Mapping both the F and R strands individually improves ability to detect weak peaks.

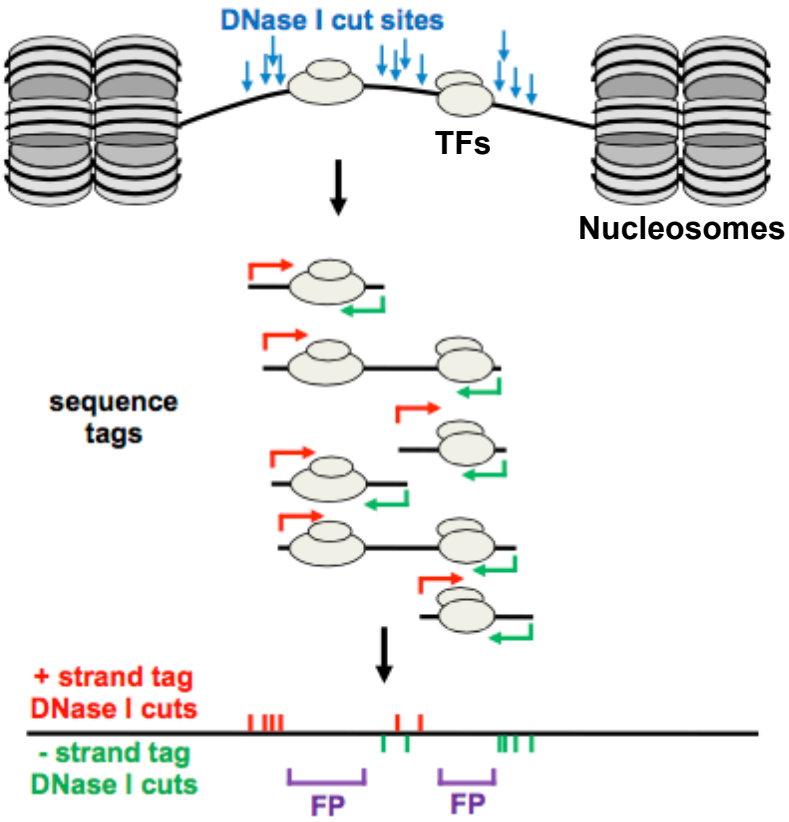


Figure 5.6 – Chromatin structure based modeling of strand-specific DNase-seq data arising from DHSs. DHSs are usually 200-250 bp across, and the DNA sub-fragments of DHSs detected by DNase-seq are typically in the order of 50 to 150 bp in length and are surrounded by nucleosomal DNA. As depicted above, most of these fragments are expected to originate from within the DHS, meaning that they are likely span the regions of DNA protected by bound factors (indicated as TFs) that give rise to footprints (FPs). This means that it is the cut site that must be used to identify FPs, and not the entire sequence tag as is used in most peak detection algorithms. Furthermore, because sequence tags represent just one end of these fragments, upper strand +ve sequence data (red arrows) should represent sequences starting upstream of these FPs, while lower strand –ve sequence data (green arrows) should represent sequences starting downstream of FPs. Wellington can identify this strand bias and accurately identify TF FPs. (Reproduced with permissions from Piper *et al.,* 2013).

Peaks uncovered in CisGenome have previously been shown to correlate with accessible regions of chromatin and are significantly more likely to contain a protein-binding motif compared to the rest of the genome (Ji *et al.,* 2011). An example of an anticipated peak profile resulting from a region of open

chromatin can be seen in the region upstream of the *RUNT-RELATED TRANSCRIPTION FACTOR 1 (RUNX1)* TSS in haemogenic endothelium (CD41+) cells (Fig 5.7). *RUNX1* plays a key regulatory role in haematopoiesis in human cells and has also been shown to drastically increase in expression between haemogenic endothelium CD41- and CD41+ cells (Lichtinger *et al.,* 2012). The peaks seen upstream of the TSS of *RUNX1* in CD41+ cells likely coincide with accessible and actively transcribed chromatin (Fig.5.7).

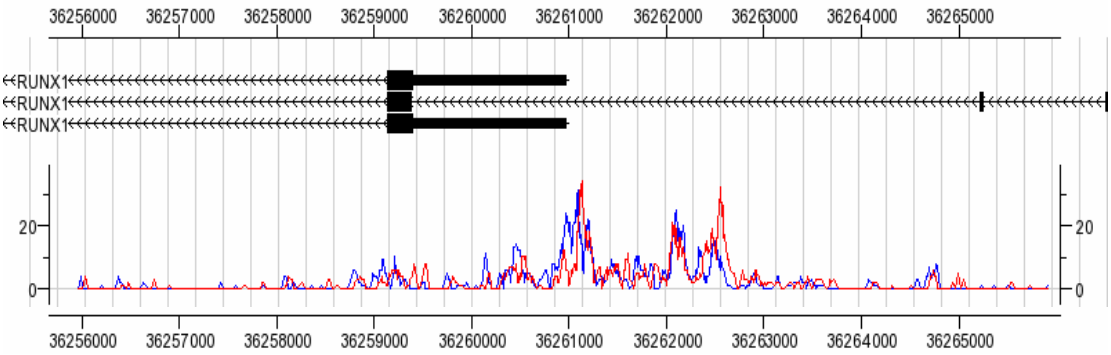


Figure 5.7 – Example of a peak profile coinciding with a region of accessible chromatin. Screenshot taken from CisGenome shows ATAC-Seq activity in a region upstream of *RUNX1* TSS in haemogenic endothelium (CD41+) cells. Region shown is Chr. 21 of the human genome, hg19, from position 36,255,801 to 36,265,891. (Generated by Katherine Woolley-Allen (University of Warwick))

Fig.5.7 shows the peak signals due to areas of open chromatin relative to spikes from noise in the data. The amplitude of peaks compared to noise is visibly distinguishable, whereas, ATAC-Seq data in this study showed no peaks along the genome that were increased in amplitude compared to noise in the data (Fig.5.8). Showing spikes in this study were most likely not biological peaks but just noise. Moreover, the low reproducibility of the spikes between the replicate sets (ZT4.2 and ZT4.3 or ZT10.2 and ZT10.3) further substantiates that these spikes are more likely noise than due areas of open chromatin (Fig.5.8).

The region seen in Fig.5.8 is representative of all regions viewed throughout the genome. The region upstream of the *EARLY FLOWERING 3 (ELF3)* TSS, *ELF3* is a component on the central circadian oscillator and is known to be a component of the evening complex. During long day conditions (16 h light: 8 h dark), at ZT

4 (early morning) *ELF3* expression levels are lowest, whereas at ZT 10 (late morning) *ELF3* expression levels are increased and expression gradually increases and peaks at ZT 16 in anticipation for the night phase (Diurnal: Mockler *et al.,* 2007). Therefore, the promoter region of this gene was predicted to be differentially fragmented in ATAC-Seq between the two time points due to different levels of chromatin accessibility or/and TF occupancy between ZT 4 and ZT 10 samples. However, this was not the case, between the two sampling times no clear differences were observed and there was as much variation between replicates as there is between sampling times (Fig.5.8). Again, this further confirms the spikes observed in this data to be due to noise.

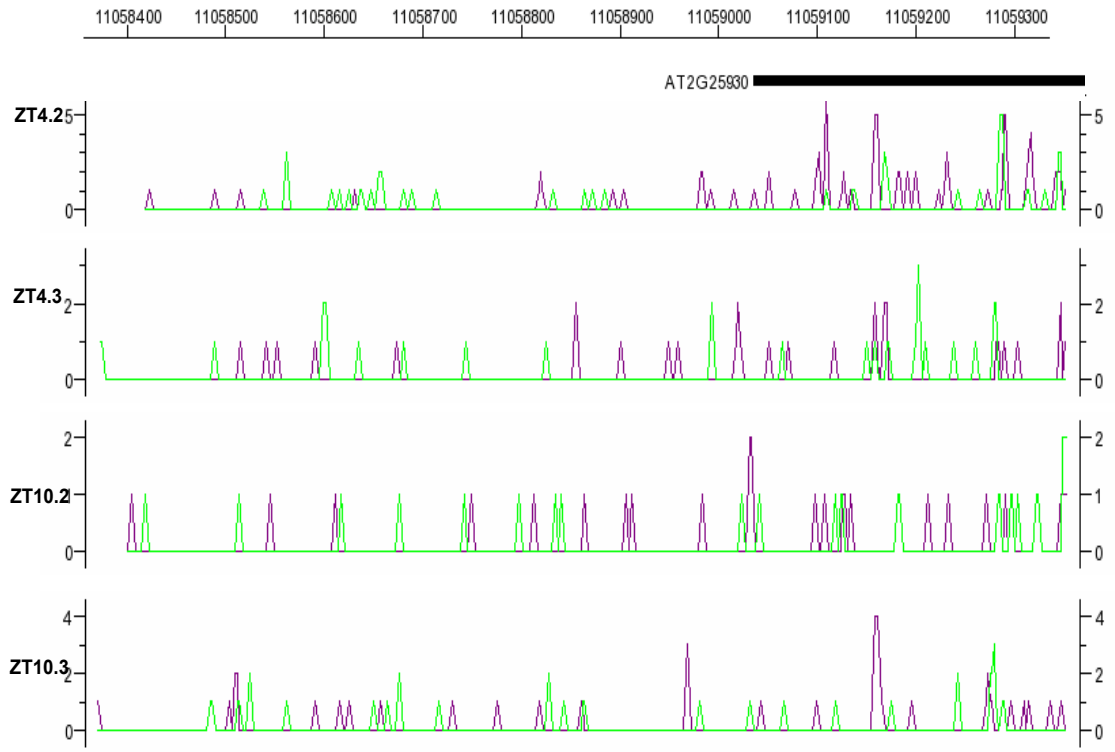


Figure 5.8 – Spikes upstream of a circadian clock gene TSS. Screenshot taken from CisGenome shows ATAC-Seq activity in a region upstream of the *EARLY FLOWERING 3 (ELF3)* transcription start site in 4-week-old Arabidopsis leaves harvested either four or ten hours after dawn (ZT 4 and ZT 10 respectively). Region shown is chromosome 2 of the Arabidopsis genome, tair9, from position 11058400-11059300. Purple – F reads and green – R reads.

Given that this data varies from the expected peak data it shows the Tn5 transposase employed in this protocol was not fragmenting as was anticipated. As this was the first time ATAC-Seq had been performed on Arabidopsis there

were many speculative reasons as to why this could be and in order to repeat this experiment successfully these potential reasons needed to be explored. The first question being was this an experimental or biological issue? Could it be that the Tn5 transposase behaves differently in plants? That perhaps Arabidopsis is somehow resistant to the action of Tn5? Perhaps the structure of the Arabidopsis nucleus renders it impermeable to the transposase? It could even be that there are certain proteins present in Arabidopsis that inhibit the Tn5 activity?

Or it is possible that the Tn5 transposase cuts Arabidopsis chromatin in the same way as it has been shown to cut Drosophila (Davie *et al.,* 2015), human (Buenrostro *et al.,* (2013); Lara-Astiaso *et al.,* (2014); Prescott *et al.,* (2015)) and 20 other mammal genomes (Villar *et al.,* 2015) and the reason the data does not look as anticipated is due to the experimental protocol? Apart from the nuclear extraction step, the experimental protocol employed in this study was optimized for human cell lines (adapted from: Buenrostro *et al.,* 2015) and given the differences between plant and human cells it could be that this protocol is not suited to Arabidopsis cells? Perhaps the transposase concentration or incubation time was too high or low? Perhaps the nuclear extraction method used damaged the chromatin? Maybe the reagents employed in the nuclear extraction inhibited the Tn5 transposase? To answer these questions and optimize the protocol the data was further analysed.

5.3.4 Troubleshooting the protocol

5.3.4.1 The Tn5 transposase was active

5.3.4.1.1 Fragment length distribution differs between plant and mammalian ATAC-Seq data

Previous ATAC-Seq studies on several eukaryotes (for example; *Drosophila* (Davie *et al.,* 2015), human (Buenrostro *et al.,* (2013); Lara-Astiaso *et al.,* (2014); Prescott *et al.,* (2015)) and 20 other mammals (Villar *et al.,* 2015)) have shown the fragment length distribution (FLD) pattern of these genomes show distinctive traits due to nucleosomal packaging. Eukaryotes package chromatin in a very specific and uniform manner; 147 bp of DNA is tightly wrapped

around a nucleosomal core, followed by between 10-90 bp of 'linker' DNA (Richmond and Davey, 2003). The wrapping around the nucleosome is helical and rotates with every ten bp (termed 'rotational positioning') (Segal *et al.,* 2006). This leaves very specific FLD patterns when looking at ATAC-Seq data.

ATAC-Seq on the human genome shows a periodicity in FLD, with peaks at every ~200 bp. This reflects the linker regions between nucleosomes being exposed and cut by the Tn5 transposase (Fig.5.9-A). Only one ATAC-Seq dataset has been published using a plant genome and Wilkins *et al.,* (2016) found that the *O. sativa* genome did not show the ~200 bp periodicity (Fig.5.9-B). Wilkins *et al.,* (2016) postulated this could be due to the structure of plant promoters being much more dense than that of mammals.

Moreover, data on human cell lines showed peaks every ~10 bp, this is expected due to the rotational positioning of DNA on nucleosomes (Fig.5.9-A). It is difficult to see if this 10 bp periodicity was observed in the *O. sativa* genome (Fig.5.9-B).

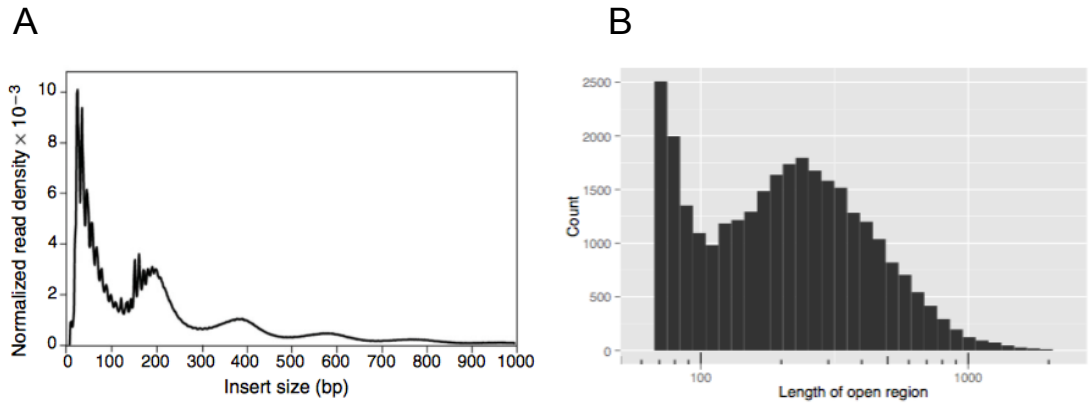


Figure 5.9 – Fragment length distribution in ATAC-Seq data differs between plant and mammalian cells. Genomes of mammals including humans have shown a ~200 bp periodicity in fragment length distribution due to nucleosomal positioning (A) (Reproduced with permissions from Buenrostro *et al*., 2015). However, this ~200bp periodicity was not seen in ATAC-Seq data of the *O. sativa* plant genome (B) (Reproduced with permissions from Wilkins *et al.,* 2016).

The FLDs of samples were plotted to see how they compared with both human and plant ATAC-Seq FLD patterns. This would give an indication of the transposase activity.

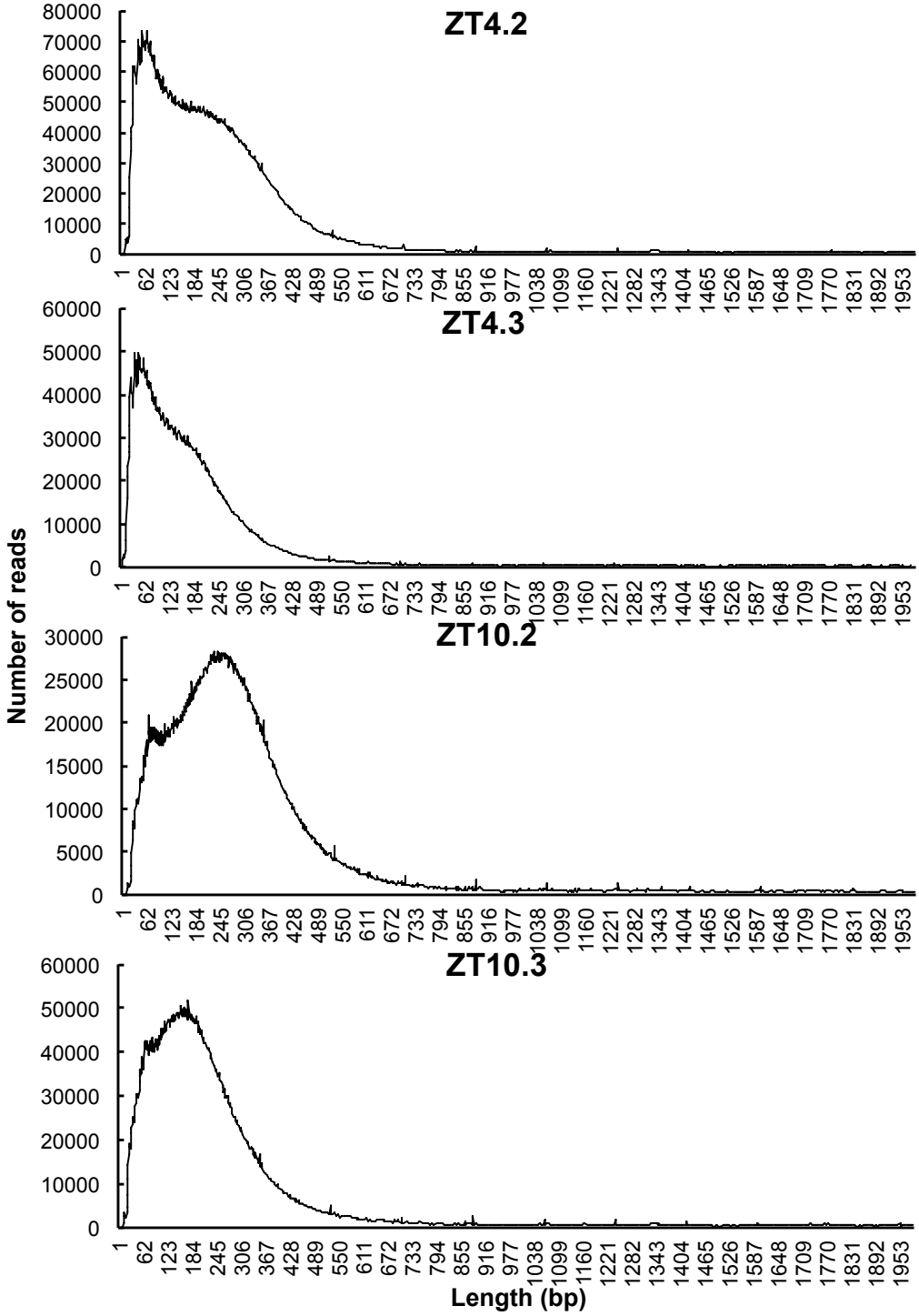


Figure 5.10 – Fragment length distribution of Arabidopsis ATAC-Seq data showed no traces of the anticipated ~200bp periodicity. ~200bp periodicity is expected in FLD due to nucleosomal positioning along chromatin. 4-week old leaves were harvested either four or ten hours after dawn (ZT 4 and ZT 10 respectively) and underwent ATAC-Seq. Reads that mapped exclusively the nuclear genome with a MAPQ score of above 22 were then analysed for fragment length distribution. The 200bp FLD periodicity observed in previous datasets was not visible here.

Each sample showed a slightly different FLD, but all samples showed the majority of reads to be less than 500 bp in length (Fig 5.10). This agreed with what was seen when ATAC-Seq carried was out on human cell lines (Fig 5.9-A). Both human and plant cell lines showed a peak in fragments below approximately 100 bp (Fig.5.9), this was observed in samples harvested at ZT 4, that have a slight peak in fragments below 120 bp (Fig.5.10). However, those harvested at ZT 10 show a much-decreased peak in these shorter fragments (Fig.5.10).

The ~200 bp periodicity observed in human genomes (Fig.5.9-A) was not evident in data from this study (Fig.5.10). Given that this periodicity has been absent in both the *O. sativa* and Arabidopsis ATAC-Seq studies carried out independently it is possible this is truly a biological phenomenon in plants.

Next the data was mined for ~10 bp peaks caused by the rotational positioning of DNA around nucleosomes. Peaks every ~10 bp can be made out in the shorter fragments, below approximately 70 bp (Fig.5.10). As such all reads with a length below 70 bp were selected and the FLDs plotted (Fig.5.11). Samples harvested at ZT 4 displayed clear peaks every ~10 bp (Fig.5.11). However, those harvested at ZT 10 showed only hints of peaks in the very short fragments and these peaks were then dampened in the longer fragments (Fig.5.11).

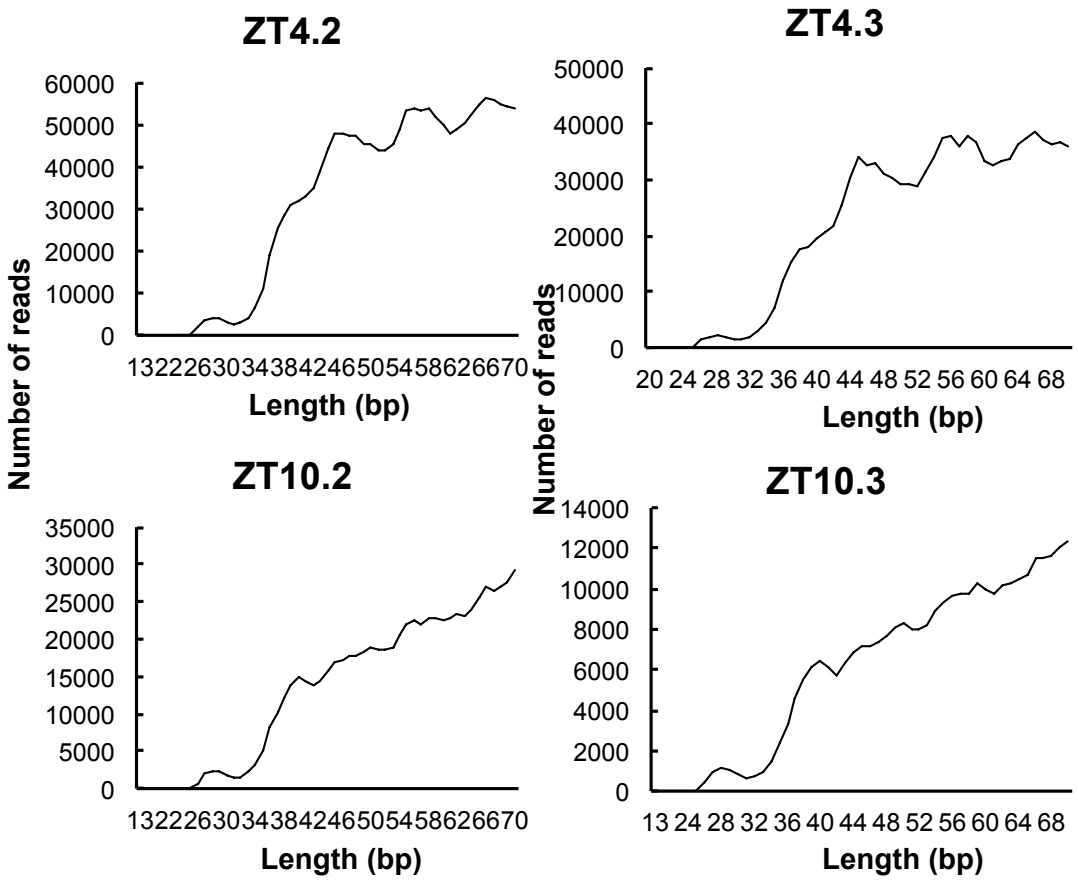


Figure 5.11 – Fragment length distribution of Arabidopsis ATAC-Seq data shows traces of the anticipated ~10bp periodicity in samples harvested at ZT 4. Peaks every ~10 bp were expected if Tn5 treatment was successful due to the rotational packaging of chromatin around a nucleosome. 4-week old leaves were harvested either four or ten hours after dawn (ZT 4 and ZT 10 respectively) and underwent ATAC-Seq. Reads that mapped exclusively the nuclear genome with a MAPQ score of above 22 were then analysed for fragment length distribution, this figure displays only fragments that are below 70 bp.

The ~10 bp periodicity can be seen in shorter reads of samples harvested at ZT 4 and the overall pattern of FLD in samples harvested at ZT 4 matched previous ATAC-Seq data from research on *O. sativa* (Fig.5.9-B). Hence, it appeared the Tn5 transposase was active during treatment of samples harvested at one timepoint (ZT 4), potentially both.

5.3.4.1.2 A Tn5 cutting bias is evident in the data

The Tn5 transposase used in ATAC-Seq to 'tagment' chromatin has previously been shown to have a cutting bias, with a preference for cutting areas enriched

for guanine (G) and cytosine (C) (Green *et al.,* (2012); Goryshin *et al.,* (1998)). This transposase has also shown preference towards specific combinations of nucleotides (nts), for example Tn5 is significantly more likely to insert into nine bp sequences with a G at the first nt position and a C at the ninth nt position (this C corresponds to a G on the complementary strand) (Fig.5.12-A) (Green *et al.,* 2012).

This insertion preference is likely to influence the reliability of the data as the transposase insertions are introducing bias into the cutting events, this is especially problematic for GC-rich genomes. However, at this stage the bias can be used to the researchers advantage. The FastQC quality checks of the sequencing data generated a per base sequence content report, in ATAC-Seq data files this report has shown sharp spikes in the first and ninth nt position for a G and C respectively, corresponding to the cutting bias of the Tn5 (Fig.5.12 – B).

Both mouse and human genome data sets have shown these spikes due to the Tn5 bias (Fig.5.12-B) and the data generated in this study on the Arabidopsis genome also shows these spikes illustrating the Tn5 cutting bias (Fig.5.12-C). Not only are the spikes in all three organisms present and in the same position, they also occur at a similar magnitude (Fig.5.12-B and C).

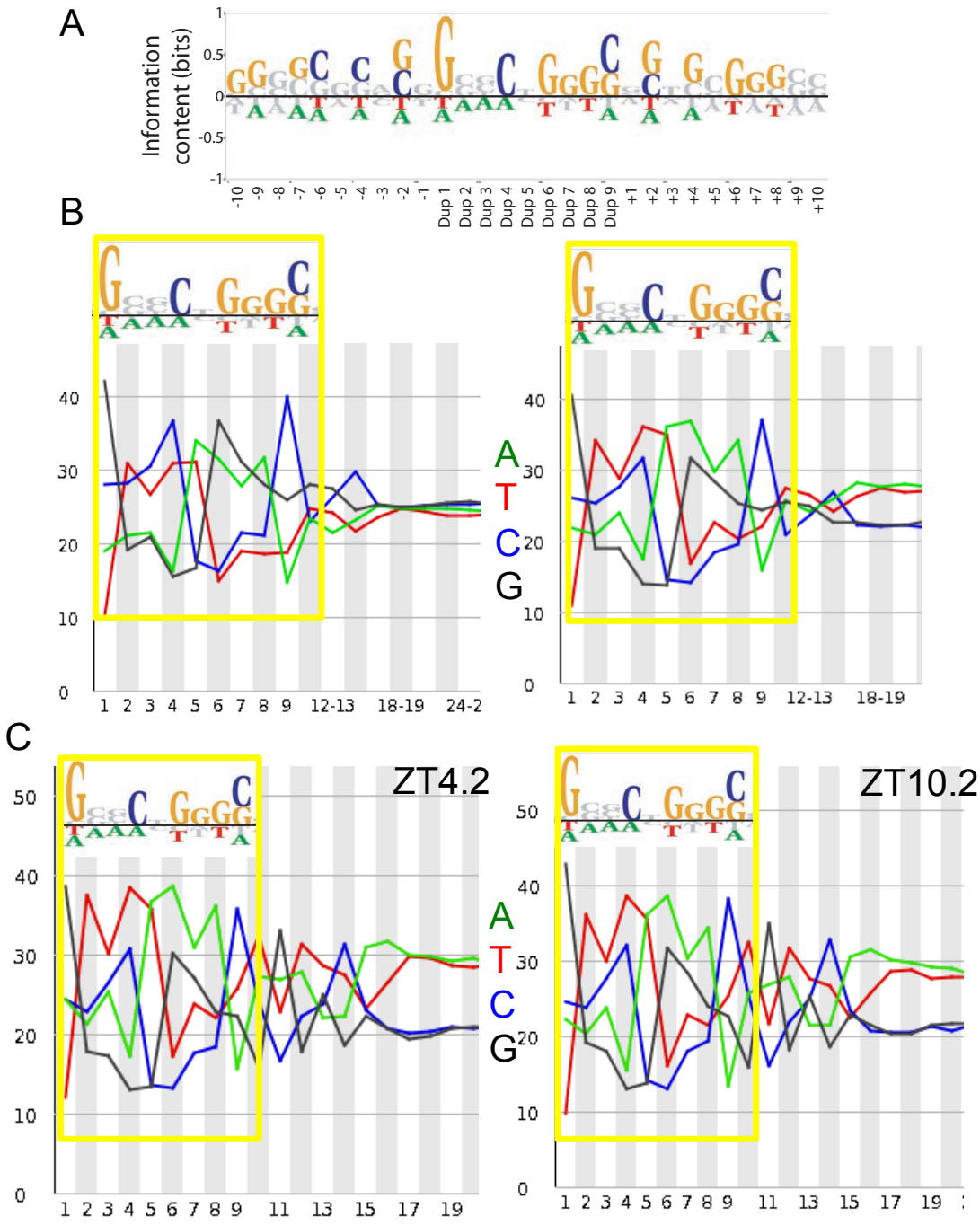


Figure 5.12 – The anticipated Tn5 cutting bias can be seen in Arabidopsis samples. Tn5 has been shown to preferentially insert and fragment sequences with a G at position 1 and a C at position 9 (A (Reproduced with permission from Green *et al.,* 2012)). This cutting bias is evident in both mouse and human ATAC-Seq data (B). The cutting bias of Tn5 can also be seen in all four Arabidopsis samples in this data (two shown) (C). The y-axis in B and C represents the percentage of reads with each nucleotide (black-G, green-A, blue-C, red-T), and the x-axis indicates the position of the base. Yellow box outlines the start and end of the Tn5 cutting bias region with the nine bp cutting motif from (A) included.

The presence of this insertion and fragmentation bias in the dataset shows the Tn5 transposase was active and responsible for the fragmentation observed in Fig.5.10 and Fig.5.11 in both ZT 4 and ZT 10 samples. The Arabidopsis genome is therefore vulnerable to cutting by the Tn5 transposase. The nuclear isolation protocol employed does not contain any inhibitory reagents stopping the action of the Tn5 transposase. Moreover, the magnitude of this bias when compared to other datasets is highly similar (Fig.5.12). This shows the proportion of reads resulting from a Tn5 cutting event rather than random shearing was comparable to other successful ATAC-Seq datasets. From here, it can assumed that the concentration of the Tn5 transposase was not too low, if it were too low the proportion of reads resulting from Tn5 cutting events would be lower than what is observed in other datasets.

Now that it has been established that the Tn5 was active and fragmenting as would be expected, the next question to ask is: was the chromatin in the expected conformation when transposase treatment occurred? If the chromatin conformation was as anticipated and the Tn5 was active then peaks from accessible chromatin regions would have been visible.

5.3.4.2 Transposase action was not influenced by chromatin accessibility

Given that peaks in reads aligning to the genome indicate accessible nucleosome free chromatin, the data was visually mined for peaks. Fifty predicted promoter regions were investigated for peaks in reads aligning to the genome and only one faint trace of a peak that may not be noise was detected. This peak was present ~400 bp upstream of the *LHY* (*LATE ELONGATED HYPOCOTYL)* TSS in ZT4.2 and hinted at in ZT4.3 (Fig.5.13). However, this peak was not observed in samples harvested later in the day at ZT 10 (Fig.5.13). This is a very faint peak and it is possible this peak is an artefact rather than due to a region of accessible chromatin. However, when compared to the peak seen upstream of the *RUNX1* TSS it can be seen this peak is highly similar. Both exhibit two distinctive peaks in the data, one in the + strand and one in the - strand and both peaks are of a similar magnitude (Fig.5.13).

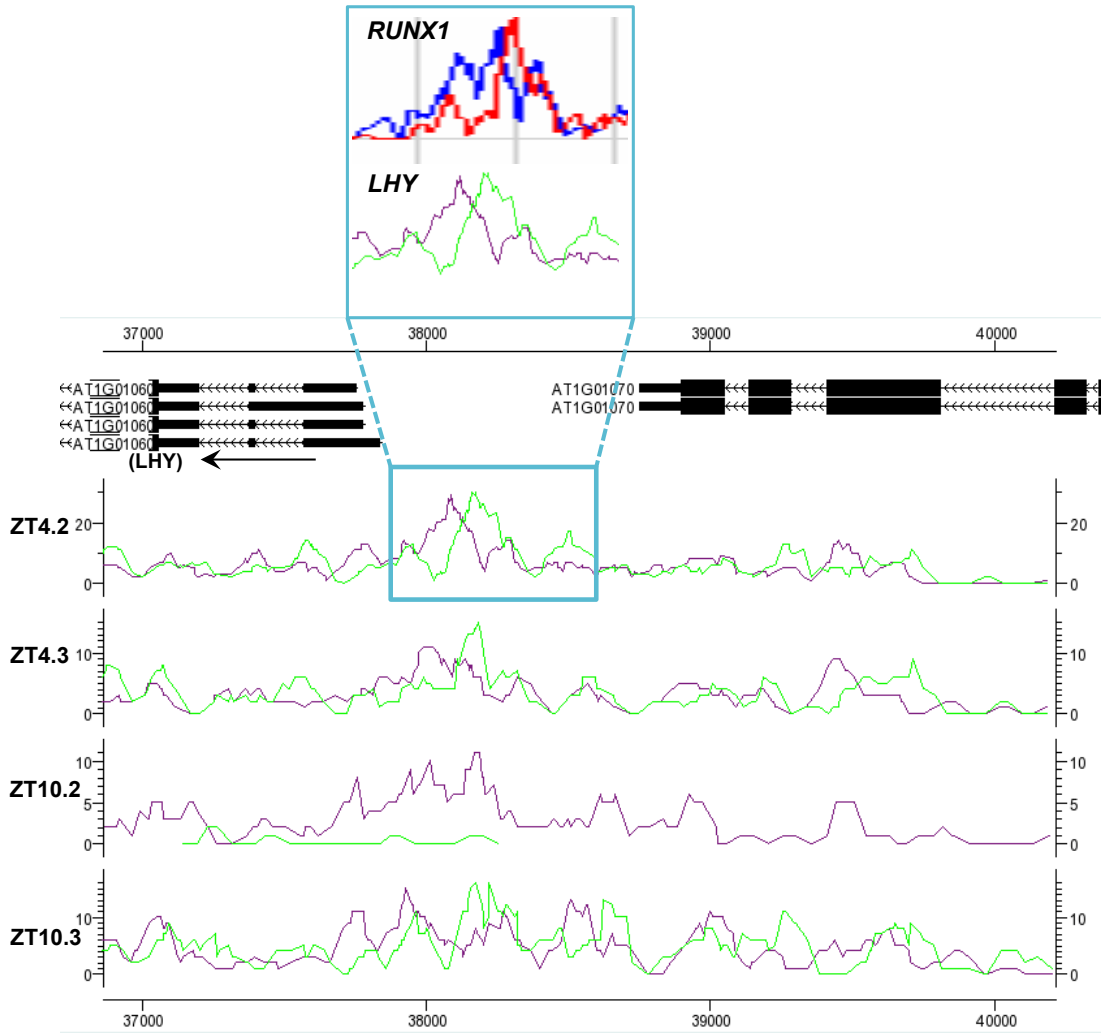


Figure 5.13 – Samples harvested at ZT 4 display a potential peak profile caused by increased chromatin accessibility upstream of the *LHY* TSS. Screenshot taken from CisGenome shows ATAC-Seq activity in a region upstream of the *LATE ELONGATED HYPOCOTYL (LHY)* TSS in 4-week-old Arabidopsis leaves harvested either four or ten hours after dawn (ZT 4 and ZT 10 respectively). Region shown is chromosome 1 of the Arabidopsis genome, tair9, from position 37900-38400. Purple – forward reads and green – reverse reads. Black arrow indicates the direction of transcription. Blue box in the ZT4.2 sample track outlines a potential peak due to increased chromatin accessibility. This is then enlarged above the track and compared to the peak observed upstream of the *RUNX1* TSS from Fig.5.7 (blue – forward, red – reverse tracks).

Full information on the function of the *LHY* gene can be found in Chapter 1. In brief, *LHY* acts redundantly with *CCA1* during the morning phase to repress evening genes within the circadian clock and other genes throughout the rest of the genome (Carre & Veflingstad, 2013). *LHY* expression peaks at ZT 2 (Carre & Veflingstad, 2013). A peak in a sample harvested at ZT 4 but no peak in samples harvested later in the day (at ZT 10) could therefore indicate chromatin

upstream of the gene early in the day is accessible and open, which increases *LHY* expression. To investigate the validity of this DNase-seq data in the region upstream of the LHY TSS was investigated (Fig.5.14). It can be seen the approximate region of the peak observed in Fig.13 correlates with the peak region observed in DNase-seq data (Fig.5.14). The peak data displayed in Fig.5.13 in sample ZT4.2 and ZT4.3 is therefore likely to be due to chromatin being more accessible.

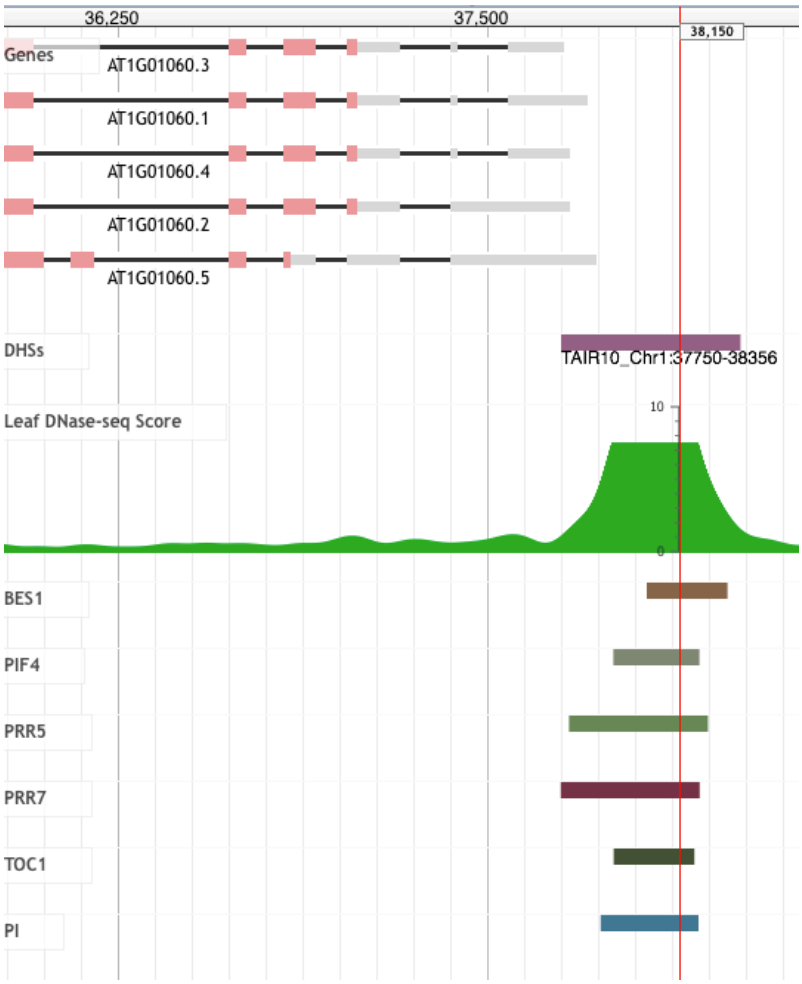


Figure 5.14 – DNase-hypersensitive site upstream of the *LHY* TSS. Screenshot taken from Plant DHSs Database (plantdhs.org: Zhang *et al.,* 2015) shows DNase-seq activity in a region upstream of the *LATE ELONGATED HYPOCOTYL (LHY)* TSS in Arabidopsis leaves. Region shown is chromosome 1 of the Arabidopsis genome, tair10, from position 36000-38500. Purple area indicates area of DNase hypersensitivity (open chromatin). This purple area is derived from the peak profile of the DNase treatment (as seen in green). Below the peak profile motifs corresponding to the peak location can be seen. The red line corresponds to the approximate location of the potential peak observed in ATAC-Seq data (Fig.5.13).

Apart from the hint at a peak upstream of the *LHY* TSS (Fig.5.13), no other peaks suggestive of an area of open chromatin were observed. This suggests that although the Tn5 transposase was active and successfully tagmented the chromatin (as previously evidenced), the chromatin was not in the expected conformation. Had chromatin been in the anticipated conformation and been tagmented, peaks of reads would be visible in areas of open chromatin due to lack of nucleosomal occupancy. However, spikes along the genome are seemingly random with very little difference between areas of predicted open and closed chromatin and only one peak seems to be potentially attributed to an area of increased chromatin accessibility (Fig.5.13). Examples of these random spikes can be seen in Fig.5.15. *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* would be expected to have regions of open chromatin in the samples harvested at ZT 4 and this should be visible from peaks. However, no such peaks were observed upstream of the *CCA1* TSS in any samples (Fig.5.15). Spikes seen were all likely due to noise and none of them display the expected peak profile (Fig.5.7).

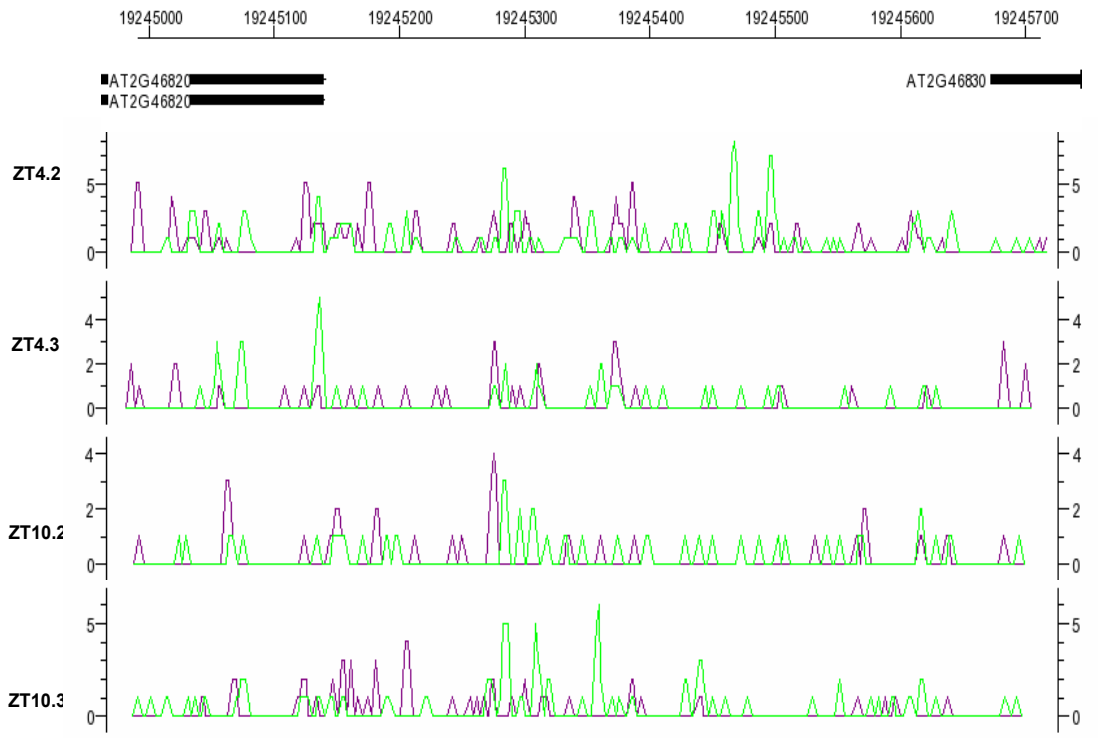


Figure 5.15 - Spikes in data are a result of noise. Screenshot taken from CisGenome shows ATAC-Seq activity in a region upstream of the *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* transcription start site in 4-week-old Arabidopsis leaves harvested either four or ten hours after dawn (ZT4 and ZT10 respectively). Region shown is chromosome 2 of the Arabidopsis genome, tair9, from position 19245000- 19245700. Purple – forward reads and green – reverse reads.

The lack of evidence of preferential cutting of open chromatin suggests chromatin conformation was somehow altered prior to transposase treatment. The nuclear extraction procedure employed in this protocol, which due to the plant cell wall differed from previous studies (Buenrostro *et al.,* 2015), could have been responsible for the observed disruption.

5.3.4.3 Transcription factor binding did not have an effect on Tn5 transposase action

Tn5 transposase was shown to be responsible for the fragmentation observed in the reads, however, these reads did not display the expected peaks associated with lack of nucleosomal occupancy in the genome suggesting an altered chromatin conformation prior to Tn5 transposase treatment. Although chromatin structure was likely altered that is not say that all TFs were removed

prior to Tn5 transposase treatment. Perhaps some TFs 'clung on' during disruption of the chromatin. To investigate this hypothesis, reads were subject to average footprinting.

TF binding motifs have been shown to be abundant throughout the genome and generally each TF has been shown to preferentially bind to specific sequences, known as TF binding motifs (Dror *et al.,* 2015). It was assumed that if the nucleosomal structure was disrupted, TFs might have been freely able to bind to TF binding motifs, as all chromatin would be accessible. Given this, a high level of TF binding motif occupancy was predicted. To enhance the likelihood of TF occupancy being detectable, motifs located within 300 bp upstream of TSS were employed. Average footprinting was used, as even if only half of the specified TF binding motifs throughout the genome were occupied, this occupancy level would be detected using average footprinting. The binding of TFs to this specific motif region leaves a distinctive footprint when the genome undergoes ATAC-Seq.

Footprinting using Wellington (Piper *et al.,* 2013) allows ATAC-Seq and DNase-seq data analysis to not only rely on peak finding but also on data derived from raw tag counts that correspond to enzyme or transposase activity at each bp. From this cutting information, Wellington can identify genomic footprints, which correspond to TF binding events (Fig.5.16 – A). Examples of footprints from DNase-seq (Fig.5.16-B) (Piper *et al.,* 2013) and ATAC-Seq (Fig.5.16-C) (Scott *et al.,* 2016) treatments can be seen. Footprints vary very little between the two DNase-seq and ATAC-Seq fragmentation methods due to the similarities of the protocols (reviewed in Tsompana and Buck, 2014).

Footprints exhibit a strand imbalance in cutting in both ATAC-Seq and DNase-seq datasets (Fig.5.16). A higher number of reads are seen on the F strand and R strand before and after TF occupancy respectively. The region of binding then has a depletion in read numbers due to the TF protecting DNA from fragmentation (Fig.5.16). The strand dependent cutting imbalance was not observed in footprints of all TFs, however it was highly abundant and never was

the reverse strand imbalance seen i.e. the F strand never peaked after the R strand (Piper *et al*, 2013)

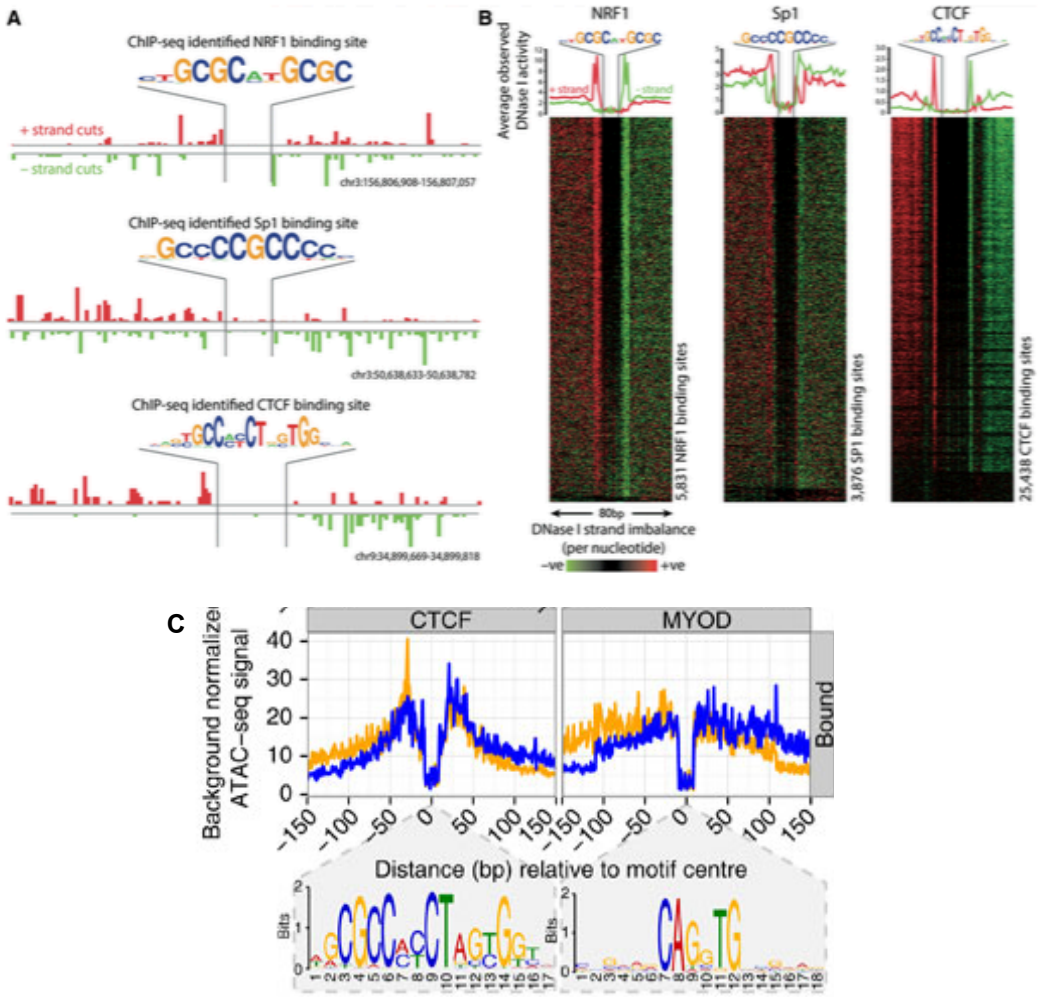


Figure 5.16 – Examples of TF footprints from DNase-seq and ATAC-Seq datasets. Single bp cuts in DNase-seq data of regions flanking a TF bound region (red = + strand, green = - strand) show DNase displays a strand bias (A – Adapted from Piper *et al.* (2013)). This strand bias can be exploited to reveal TF bound regions of chromatin in DNase-seq datasets (B – Adapted from Piper *et al.* (2013)) and ATAC-Seq datasets (C (yellow = + strand, blue = - strand) – Adapted from Scott *et al.* (2016)). (Permissions obtained from both Piper *et al.* (2013) and Scott *et al.* (2016)).

Given the single bp definition achievable using Wellington it was hypothesized this could detect TF binding events. As such, footprinting was carried out in regions of known binding motifs where TF occupancy would be anticipated. Ten motifs were randomly selected from a range of TF families from Franco-Zorrilla *et al.,* (2014) and Weirauch *et al.,* (2014). These motifs included binding sites

for the TFs; CCA1, ANAC58, DEAR3, KAN1, MYB52, RAP2.3, MYC2, RVE1, TCP23 and WRKY18. Once motifs were selected their locations throughout the genome were determined and all the coordinates that corresponded to regions within 300 bp of a TSS were analysed. This analysis revealed the average cut profile for each bp in the motif sequence throughout the reads in each sample. It would be expected that motifs that were commonly occupied by TFs would give distinctive footprints such as those in Fig.5.16.

Footprints for all samples can be seen in white in Fig.5.17, none of which show the expected profile. A TF binding event would have higher signal intensity (as is seen in Fig.5.16). The F and R strand would be evident (again visible in Fig.5.16), the F strand would have a higher intensity approaching the binding site and a lower intensity after the binding site, whereas the R strand would have a lower intensity approaching the binding site and a higher intensity after the binding site. The expected the lack of reads within the occupied region was also not seen (Fig.5.17). A dip in intensity approaching the binding site would have been evident, as the TF would have protected the chromatin from transposase cutting (as is evidenced in Fig.5.16). Given the lack of peaks within the data as well as this evidence it appears that TF and nucleosomal occupancy did not influence the fragmentation by the Tn5 transposase.

The spikes observed in the footprint data for DEAR3 and MYC2 motifs in the full-length fragments are likely due to the bias of the Tn5 transposase cutting (Fig.5.17). Spikes are observed in areas corresponding to Gs or Cs (this GC bias was previously outlined). Areas with decreased occupancy in the CCA1 motif regions are also likely due to the Tn5 cutting bias. To correct for this bias an alternative to Wellington or an optimized version of Wellington for ATAC-Seq data should be developed.

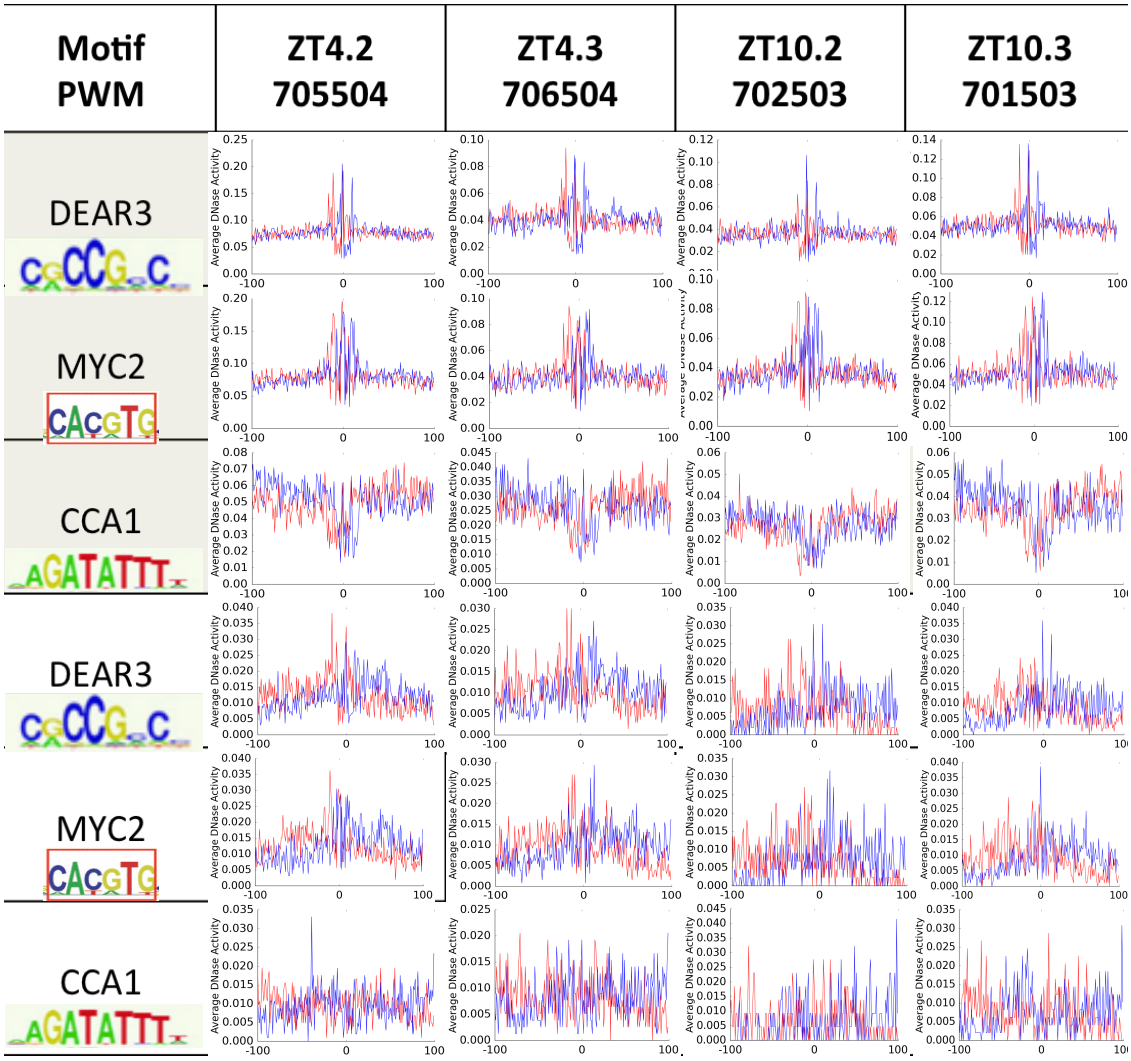


Figure 5.17 – Average footprints of commonly bound TF binding motifs across all samples revealed TFs are unlikely to be bound to chromatin in any of the four samples. The expected footprint of a region bound by a TF can be seen in Fig.5.16. These profiles do not reflect the anticipated footprint. Ten motifs were located across the Arabidopsis genome and those found to be within 300 bp upstream of TSS were employed for footprinting using Wellington (Piper *et al.,* 2013). Footprints of these regions in fragments (grey) or fragments with a length of below 70bp (white) across all four samples are displayed. Three representative protein-binding motifs can be seen (DEAR3 and CCA1 Position weight matrix (PWM) adapted from Franco-Zorrilla *et al.* (2014) and MYC2 PWM adapted from: Godoy *et al*. (2011)). y-axis displays the signal intensity derived from average number of cutting events per bp. Y-axis displays the bp within the motif and approximately 100 bp either side. Red = + strand, blue = - strand.

Investigating the FLD data it appeared fragments up to the length of 70 bp looked most similar to what is anticipated for ATAC-Seq data based on animal genomes (Fig.5.11). The FLDs displayed in Fig.5.11 the ~10 bp periodicity where as after 70 bp the 10 bp periodicity was dampened (Fig.5.10). Moreover,

it is expected shorter fragments will contain the most information in relation to *cis*-regulatory elements. Longer fragments tend to result from areas of chromatin spanning regions of nucleosomes.

As such, the shorter reads may give footprint profiles characteristic of TF binding events. Therefore, the shorter reads (>70 bp) were run through the same pipeline as above. Although the shorter fragments appear to exhibit a footprint closer to what is expected of a TF-DNA binding event, the profiles are still not typical of a TF binding event (again, this data is representative of all ten motifs) (Fig.5.17).

The shorter fragments gave footprint profiles marginally closer to what is expected of a TF binding region. This is especially true for footprints at the DEAR3 and MYC2 binding motif locations in samples ZT4.2 and ZT10.3 (Fig.5.17). Footprints from these motifs in the shorter fragments showed the previously discussed strand bias slightly more than the longer fragments (Fig.5.17). The F strand is slightly increased prior to the motif when compared to the R strand and visa-versa after the motif. Moreover, The actual motif region shows a slight depletion compared to the footprints seen from longer fragments (Fig.5.17). However, the footprint for CCA1 is very different to what would be expected if a TF binding event was occurring. These hints at footprints in the shorter reads show a few TFs may have 'clung on' after the chromatin was disrupted, however this is highly speculative.

By filtering for only short fragment lengths (>70 bp) many reads were lost (Fig.5.18) and with so few reads plotting TF binding events using footprinting is difficult. Had read numbers been greater for shorter fragments footprinting may have given improved binding profiles. However, as the profiles are there is very little evidence of any TF binding events (Fig.5.17).

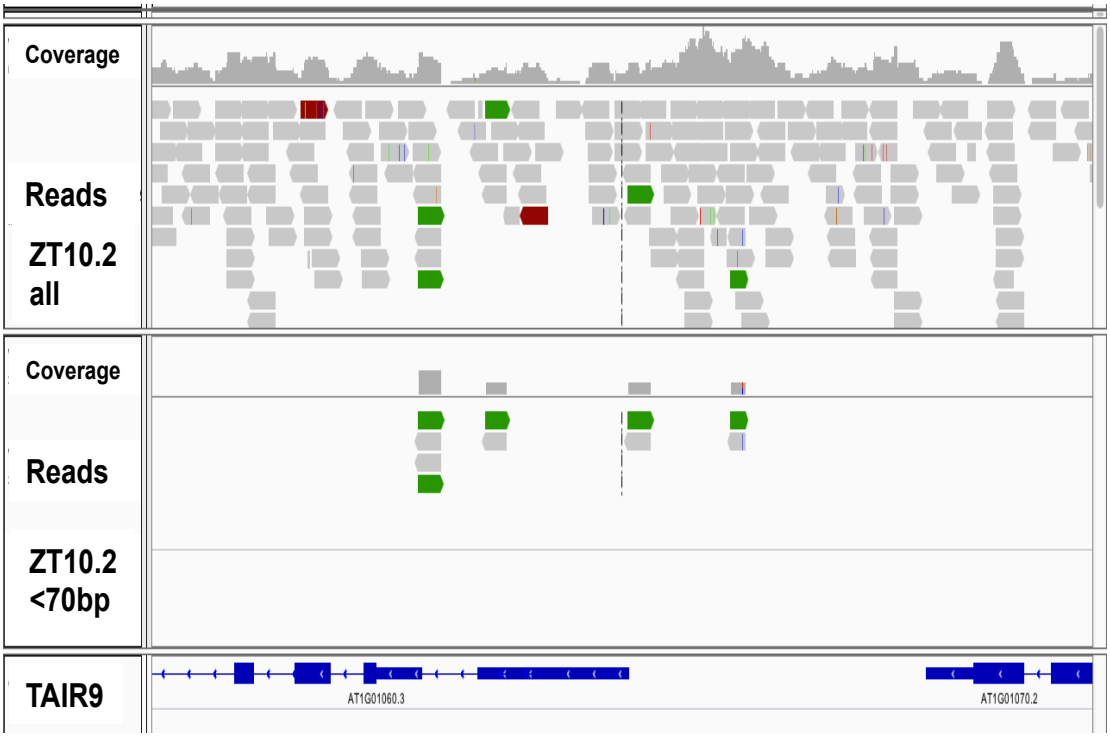


Figure 5.18 – Genome coverage is too low to carry out analysis when reads are filtered for only fragments of below 70bp in length. Screenshot taken from IGV shows read distribution in a region upstream of the *LATE ELONGATED HYPOCOTYL (LHY)* TSS in 4-week-old Arabidopsis leaves harvested ten h after dawn (ZT 10, sample: ZT10.2). Region shown is from chromosome 2 of the Arabidopsis genome, tair9. Reads at the top contain fragments of all lengths and give a relatively high coverage of the genome (read n mapping to nucleus = 7528256), whereas when reads are reduced to only fragments with a maximum length of 70 bp it can be seen that read coverage is largely reduced (read n mapping to nucleus = 1098434) and very few reads remain.

5.4 Discussion

Overall these results display that the Tn5 transposase was active and did cut the Arabidopsis chromatin (as evidenced by the Tn5 cutting bias seen in all samples (Fig.5.12)). However the chromatin was not in the expected conformation as no peaks typical of open chromatin regions can be seen in reads from any samples (Fig.5.8 & Fig.5.15) apart from the faint hint of a peak upstream of the *LHY* TSS (Fig.5.13). Moreover, no motif regions anticipated to be bound by TFs showed an average footprint that would be expected if a TF was bound (Fig.5.17).

5.4.1 The nuclear extraction procedure damaged chromatin conformation

ATAC-Seq has been praised as a quick and relatively easy protocol to implement and optimize compared to other chromatin mapping techniques, such as DNase-seq (advantages reviewed in Tsompana and Buck, 2014). Previous studies on eukaryotic organisms including Drosophila (Davie *et al.,* 2015), humans (Buenrostro *et al.,* (2013); Lara-Astiaso *et al.,* (2014); Prescott *et al.,* (2015)) and 20 other mammals (Villar *et al.,* 2015) have given excellent results and allowed mapping of binding regions along these genomes. However, the results in this study have shown that implementing ATAC-Seq on plants may not be as simple and easy as previously thought.

The experimental protocol employed was optimized for human cell lines (adapted from: Buenrostro *et al.,* 2015), which lack a cell wall. However, plant cells have a tough cell wall as well as a cell membrane. Given this the nuclear extraction step prior to Tn5 treatment differed between the Buenrostro *et al.,* (2015) protocol and the one employed for plant cells. However, steps following the nuclear extraction were highly similar to the steps employed by Buenrostro *et al.,* (2015).

This protocol difference as well as the strong evidence suggesting that although the Tn5 transposase was active and working as anticipated the chromatin was not in the correct conformation upon transposase treatment suggests the nuclear extraction step is where the chromatin structure was damaged.

5.4.1.1 Grinding fresh leaf tissue disrupted chromatin conformation

Wilkins *et al.,* (2016) carried out ATAC-Seq on plant leaf cells of *O. sativa* and the data showed Tn5 had a cutting bias towards areas of open chromatin (Appendix 8). All steps by Wilkins *et al.,* (2016) after the nuclear extraction were similar to the ATAC-Seq protocol employed in this chapter. However the sampling and nuclear extraction steps varied. Given this it appears the nuclear extraction protocol employed by Wilkins *et al.,* (2016) is more suited to ATAC-Seq experiments, as it does not appear to disrupt the conformation of chromatin within the nucleus whereas the protocol implemented in this study did.

Wilkins *et al.,* (2016) flash froze and ground tissue with liquid nitrogen prior to extracting nuclei, however, research in this chapter did not. Wilkins *et al.,* (2016) gained insight into chromatin accessibility from data retrieved after ATAC-Seq on nuclei that had been previously flash frozen. This is unexpected given previous results from ATAC-Seq research on human motor neurons showing samples that had been flash frozen gave noisy data when compared to fresh samples due to the freezing process impairing chromatin structure and damaging nuclear integrity (Milani *et al.,* 2016). Also the widely adhered to ATAC-Seq method developed by Buenrostro *et al.,* (2015) advises the use of fresh tissue.

It is possible plant cells are more resistant to the damage caused by freezing than mammalian cells due to the presence of a cell wall and that flash freezing and/or grinding tissue with liquid nitrogen as part of the nuclear isolation has no influence on the results of mapping chromatin accessibility in plants. Or it could even be the case that flash freezing the tissue keeps the nuclei and chromatin in a more conserved state than grinding fresh tissue. Wilkins *et al.,* (2016) ATAC-Seq data in rice, DNase-seq and ChIP-seq in rice (Zhang *et al.,* 2012), DNase-Chip assays in Arabidopsis (Shu *et al.,* 2013) and DNase-seq in Arabidopsis (Cumbie and Filichkin *et al.,* 2015) all gave chromatin accessibility information from plant cells after tissue had been ground with liquid nitrogen

to assist in the nuclear extraction process. However, Sullivan *et al.,* (2014) carried out DNase-seq on fresh Arabidopsis tissue and gained high-resolution chromatin accessibility data allowing TF occupancy to be seen.

Sullivan *et al.,* (2014) used biotin tagged isolation of nuclei tagged in specific cell types (INTACT) (Deal and Henikoff, 2010) nuclei lines coupled with a razor chopping method followed by triple filtering through nylon mesh to get a liquid which purified nuclei were extracted from using streptavidin affinity reagents. This method gently mechanically disrupts plant tissue structures and yields intact nuclei. It is possible that the method employed in this chapter (grinding fresh tissue with the nuclear extraction buffer in a pestle and mortar) to disrupt plant cell structures was too vigorous and damaged the chromatin conformation.

To optimize this step in future studies it is advised plant tissue is either flash frozen and ground with liquid nitrogen as was performed in Wilkins *et al*. (2016). Or given the unknown effect low temperatures could have on the results, it is advised that INTACT (Deal and Henikoff, 2010) biotin tagged nuclear plant lines are employed and nuclei are extracted as outlined in (Sullivan *et al.,* 2014).

However, even with these optimization steps the data would only be of an equal quality to Wilkins *et al.,* (2016), which could be used to identify areas of open chromatin when all samples were pooled but areas of open chromatin between stress treated samples could not be identified due to low read coverage. Low read coverage of the nuclear genome was obtained as ~70% of reads aligned to the chloroplast genome.

5.4.2 The chloroplast and mitochondrial genomes are highly susceptible to fragmentation by Tn5

Arabidopsis cells contain three genomes, the nuclear genome, the mitochondrial genome and the plastid genome. This chapter showed ~80% of reads mapped to the chloroplast genome in Arabidopsis ATAC-Seq studies

(Fig.5.5) and Wilkins *et al.,* (2016) reiterated this problem by finding ~70% of reads mapped to the chloroplast genome in rice ATAC-Seq studies. Differences between this chapter and Wilkins *et al.,* (2016) include variations between protocols, species differences and differences in the tissue ages, however, independent of these factors both data show a disturbingly high level of reads mapping to the chloroplast genome.

Moreover, approximately 10% of reads are lost in this data as they aligned to the mitochondrial genome (Fig.5.5) and 15% of reads in the rice genome also mapped to the mitochondrial genome (Wilkins *et al.,* 2016). Previous ATAC-Seq studies have shown 10-50% of reads in mammalian cells align to the mitochondrial genome (Buenrostro *et al.,* 2015).

Given these high read alignment rates it would be expected that the tissue employed in this study contained more chloroplast DNA than it did nuclear DNA and an approximately equal amount of mitochondrial and nuclear DNA. However, previous research has shown the ratio of plastid to nuclear DNA varies between 0.4%-20%, with the highest level observed in mature leaves (Rauwolf *et al.,* 2010). The ratio of mitochondrial to nuclear DNA is much lower and varies between 0.5%-4% (Rauwolf *et al.,* 2010). So even without enriching for nuclei the ratio of reads mapping to the chloroplast or mitochondria should at maximum be 24%. Nevertheless, in this study reads mapping to either organelle were approximately ~90%.

5.4.2.1 Tn5 has a bias towards cutting the chloroplast and mitochondrial genomes

This unanticipated enrichment of reads mapping to the chloroplast or mitochondrial genomes could be attributed to a number of reasons. It is possible that the Tn5 transposase preferentially cuts these genomes? Or it is possible that a large number of sequences in nuclear genome are also found in the mitochondrial or chloroplast genomes?

To investigate if this cutting is a mechanism of Tn5 or if it is related to the plant genomes, DNase-seq data was investigated for read numbers mapping to either the chloroplast or mitochondria (Table 5.1). The similarity of DNase-seq and ATAC-Seq protocols and data generated has been discussed, if both show high alignment rates to the mitochondrial and chloroplast genomes then the high mapping rates are not attributed to a Tn5 cutting bias. Sullivan *et al.,* (2014) carried out DNase-seq on fresh tissue from 7-day-old Arabidopsis seedlings and root cells. Sullivan *et al.,* (2014) employed a nuclear extraction technique that was similar to what was carried out in this chapter.

Organelle	Expected (Rauwolf)	DNase-seq (Sullivan)	ATAC-Seq (Wilkins)	ATAC-Seq (CS)
Nucleus	≥76%	70.50%	15%	10%
Chloroplast	0.4-20%	27.50%	70%	80%
Mitochondria	0.5-4%	1.8%	15%	10%

Table 5.1 – Mapping of reads to the chloroplast and mitochondrial genomes is more frequent in ATAC-Seq compared to DNase-seq data. The expected percentages of reads to be aligning to each plant genome show most reads should map to the nuclear genome (Rauwolf *et al.,* 2010). Most reads mapped to the nuclear genome when DNase-seq was carried out on Arabidopsis seedlings (Sullivan *et al.,* 2014). However both ATAC-Seq datasets showed most reads were aligning to the chloroplast (in rice (Wilkins *et al.,* 2016) and Arabidopsis).

In DNase-seq data on average less than 2% of reads aligned to the mitochondrial genome (Table 5.1). Sullivan *et al.,* (2014) also found that, on average, 27.5% of reads aligned to the chloroplast in samples of 7-day old whole seedlings (Table 5.1). Interestingly, the percentage of reads mapping to the chloroplast in whole seedlings varied greatly depending upon light treatments, with seedlings grown in constant darkness having on average 17% of reads aligning to the chloroplast whereas those seedlings grown in light:dark (L:D) conditions showed 34% of reads aligning to the chloroplast (Sullivan *et al.,* 2014).

It is clear from Table 5.1 that Tn5 acts differently to the DNase enzyme and the Tn5 has a bias for fragmenting both mitochondrial and chloroplast genomes over the nuclear genome. Where does this bias originate? Could it be that the membranes surrounding the mitochondria and chloroplast are more permeable

to the Tn5 enzyme? Could it be that the genome itself is 'easier' for the Tn5 to cut? Could this mechanism be related to the Tn5 being derived from bacteria and the chloroplast and mitochondria evolving from bacteria?

Tn5 is derived from the bacteria, *Escherichia coli*, and in its natural host environment acted as a Class I transposon to carry antibiotic (kanamycin, bleomycin and streptomycin) resistance throughout the genome (Bennett, 2008). Both the plant chloroplasts and the mitochondria have evolved from bacteria, endosymbiotic a-proteobacteria and cyanobacteria-like prokaryotes, respectively (Andreux *et al.,* (2013); Reyes-Prieto *et al.,* (2007)). Both organelles have retained bacterial structures such as 70S ribosomes that are more closely related to bacterial than plant ribosomes (Leaver *et al.,* (1976); Tiller *et al.,* (2012)).

The similarities between these plant organelles and bacteria have remained so strong that compounds designed to inhibit bacterial function (antibiotics) have been shown to have the off-target effect of inhibiting the function of chloroplasts and mitochondria (Cocito *et al*. (1979); Moullan *et al,* (2015)). Tetracyclines are a class of broad-spectrum antibiotics and act by inhibiting bacterial protein synthesis by blocking transfer RNA (tRNA) recruitment to the ribosome (Gossen *et al.,* 1995). This antibiotic class has been shown to severely repress the mitochondrial function, by the same ribosomal tRNA blocking mechanism (Moullan *et al.,* 2015). Moreover, another class of antibiotics, streptogramins, which also inhibit bacterial protein synthesis via the ribosome, has been shown to inhibit chloroplasmic protein biosynthesis (Cocito *et al.,* 1979).

It could be that the similarities preserved between the plant organelles and bacteria are not only causing these organelles to be susceptible to antibiotics but also the increased vulnerability to the action of other bacteria-derived compounds such as the Tn5 transposase. It is possible the chloroplast and mitochondria better reflect the host environment Tn5 has evolved to thrive in and this explains the increased genome fragmentation in both organelles

compared to the nucleus. Several potential reasons the Tn5 may act more on the chloroplast and mitochondrial genomes are outlined below and may be related to the shared lineage of these organelles with *E. coli* or may be independent of this shared lineage.

The activity of transposases such as Tn5 in the natural host environment is highly regulated and kept to a low level, as the unregulated movement of transposons throughout a genome would be hazardous to cells (Steiniger-White *et al.* 2004). This regulation is known to be complex with many factors such as host chaperones, TFs, DNA methylation contributing to the regulation of transposase activity (Kohl and Bock, 2009). Both the mitochondria and chloroplast organelles lack these regulatory mechanisms controlling transposase activity whereas the plant nucleus has many of these regulatory mechanisms. It is plausible that the presence of these regulatory factors suppresses Tn5 activity in the nucleus and the organelles that lack these regulatory mechanisms allow Tn5 to thrive.

The enzymatic reaction of the Tn5 transposase binding to and cleaving DNA is catalyzed by a co-factor, Mg^{2+} (Steiniger-White *et al.* 2004). It could be that the chloroplast and mitochondria contain a higher concentration of free magnesium (Mg^{2+}) when compared to the nucleus and this difference encourages the increased activity of Tn5 in the chloroplast and mitochondrial compartments. Indeed it has been shown that the chloroplast and mitochondria is where the majority of the adenylate kinase (AK) activity is localised and AK has been shown to mediate the Mg^{2+} concentrations (Igamberdiev and Kleczkowski, 2003). It could therefore be the situation that by providing the transposase with more co-factors in these organelles the transposase has an increased activity.

The bias Tn5 shows towards the chloroplast and mitochondrial genomes is significant. However, why this transposase might show this bias is still largely unknown and hypotheses are speculative. More research is needed to evaluate this bias.

5.4.2.2 Read losses in the chloroplast and mitochondrial genomes can be decreased

Several methods could be implemented to combat read losses to the mitochondrial and chloroplast compartments. Currently research on non-plant cell types (Buenrostro *et al.,* 2015) employ deep coverage sequencing so that read numbers are sufficient to carry out analyses on the nuclear genome even if up to 50% of reads are lost to the mitochondrial genome. However, if plant researchers also need to account for losses of up to 80% to chloroplast genomes then the depth of reads required becomes economically unviable. Generally plant genome projects aim for less than 10% of reads to be mapping to mitochondrial or chloroplast genomes (Lutz *et al*., 2011).

For human genome ATAC-Seq a read depth of 200 million reads is advised if the aim of the study is TF footprinting (Buenrostro *et al.,* 2015). Given the human genome is ~20 times the size of the Arabidopsis genome (0.157Gb : 3.3Gb), theoretically only 10 million reads would be needed to carryout TF footprinting in Arabidopsis ATAC-Seq studies.

On average ~7million reads mapped to the nucleus in this study, this was only 10% of the mapped reads when 4 samples were run on a single lane with a 200 million read depth. Therefore, only a slight increase in the read depth would be required, two samples on one lane (so ~100 million reads per sample) would suffice. Two samples per lane on runs with such depth will be costly to the researcher, especially when biological replicates and different samples are essential. Ideally the read losses could be decreased.

Several options are available to reduce read losses to these compartments rather than increasing sequencing read depth. The most obvious step being a cleaner extraction method for nuclei as the method employed in this chapter was crude. This step could be optimized and this would lead to a reduced number of both organelles being treated with the transposase. Typical nuclear extractions (including the one employed in this chapter) involve several steps; tissue disruption (grinding), filtration, centrifugation, detergent treatment to

disrupt chloroplast/mitochondrial membranes, centrifugation of samples to separate nuclei by density. The crucial step to ensure low organelle contamination is the detergent treatment, if the detergent concentration or treatment time is too high then not only will organelle membranes be disrupted but so will nuclear membranes, however if either of these factors is too low then organelle contamination will be rife. Extra steps can be included in this protocol to decrease organelle contamination and increase the purity of nuclei; a common additional step is using a sucrose density gradient followed by a low speed centrifugation to obtain pure nuclei (Sikorskaite *et al.,* 2013).

Another potential nuclear isolation method, which may be more effective in reducing organelle contamination, is the use of isolation of nuclei tagged in specific cell types (INTACT) (Deal and Henikoff, 2011). This method utilizes biotin labeled nuclei by allowing isolation of streptavidin-coated magnetic beads and yields pure nuclei. However, these biotin tagged lines may interfere with the structure of the nucleus and/or influence the action of the Tn5 transposase. This method may also be difficult to implement if studies require transgenic lines with mutations other than the biotin labeled nuclei, as the INTACT mutation will need to be introduced into the transgenic line. Creating double mutants can be time-consuming and costly.

A protocol has been developed to combat losses to the mitochondrial genome in mammalian cells. A CRISPR/Cas9 assisted removal of mitochondrial DNA (termed CARM) uses single guide RNAs (sgRNAs) to target mitochondrial DNA sequences after ATAC-Seq treatment in mouse embryonic cells (Wu *et al.,* 2016). This was shown to significantly increase the percentage of reads mapping to the nuclear genome (Wu *et al.,* 2016). The CRISPR/Cas9 system could also be used to target plant mitochondria or chloroplast DNA for Cas9 assisted removal by designing sgRNAs that map to the mitochondrial and chloroplast genomes.

5.4.3 Proposed protocol optimization for ATAC-Seq in plants

After an extensive analysis of data obtained from an ATAC-Seq experiment on Arabidopsis it can now be stated that although the data was not sufficient to identify TF binding or areas of open chromatin the data analysis did identify that two steps within the protocol need optimizing. The first step is the method of tissue disruption prior to nuclear extraction; grinding fresh leaf tissue appeared to disrupt the native chromatin conformation. To prevent this tissue could either be flash frozen (as employed by Wilkins *et al.,* (2016) for ATAC-Seq on rice) or if fresh tissue is preferred, INTACT lines with biotin tagged nuclei could be grown and tissue disrupted using a razor chopping method (as employed in Sullivan *et al.,* (2014) for DNase-seq on Arabidopsis) (Fig.5.19). The second step is required to reduce reads mapping to the chloroplast and mitochondria genomes. This could be implemented in several ways; firstly, a sucrose gradient could be employed post-nuclear extraction to obtain pure nuclei (as carried out in Sikorskaite *et al.,* (2013)). Or/and a CRISPR/Cas9 system (employed by Wu *et al.,* (2016) to reduce mitochondrial read alignments in mammalian cells) could be used to target and remove DNA fragments from chloroplast or mitochondrial DNA (Fig.5.19).

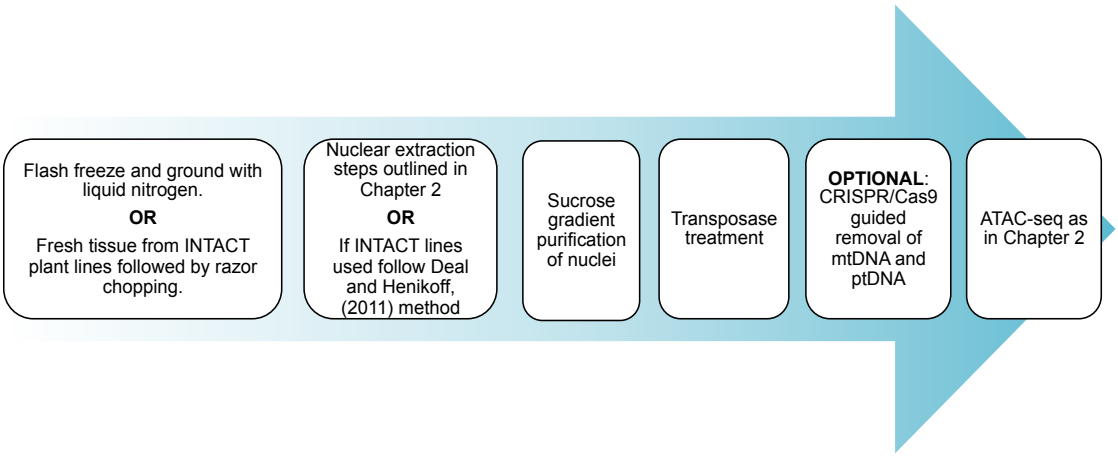


Figure 5.19 - Steps proposed to optimize ATAC-Seq on plant cells. Optimized nuclear extraction and removal of mitochondrial (mtDNA) and chloroplast (ptDNA) contamination prior to sequencing. Transposase treatment conditions should not differ from what is outlined in Chapter 2. The optional removal of mtDNA/ptDNA by Cas9/CRISPR may be laborious and expensive while this technology is still relatively new and this step may be more feasible in future. Following this subsequent amplification and clean up steps outlined in Chapter 2 should be followed.

Chapter 6: Discussion

6.1 How does the plant differentially mediate the defence response against *Botrytis cinerea* at dawn compared to night?

6.1.1 Differential regulation of the defence response against *B. cinerea* does not occur via circadian regulation of stomatal aperture

The time-of-day dependent immunity observed against the hemi-biotrophic and obligate biotrophic pathogens, *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis (Hpa)* has previously shown to be partially dependent upon the circadian regulation of stomatal aperture (Panchal *et al.* (2016); Zhang *et al.* (2013b)). Several *P. syringae* strains have evolved to manipulate the circadian regulation of stomatal aperture via the synthesis and release of coronatine (Panchal *et al.*, 2016). Therefore, the first question when investigating how the plant circadian clock mediates the defence response against *Botrytis cinerea* was whether circadian regulation of stomatal aperture played a role. Upon investigating this it was seen that *B. cinerea* does not preferentially enter plant cells via the stomatal openings under the conditions used throughout this study (Chapter 3 – Fig.3). However, it could be that *B. cinerea* acts differently when it lands on different tissue types or under different infection conditions. For example, when *B. cinerea* lands on soft fruits such as grapes it may enter tissue via stomatal openings. However, in the context of this study the circadian regulation of stomatal aperture does not appear to contribute to the outcome of the *B. cinerea* infection process.

6.1.2 The plant transcriptome is more responsive to *B. cinerea* inoculations at dawn compared to night

The results Chapter 3 suggested the transcriptome responds differently in response to *B. cinerea* inoculations at dawn or night, with 901 genes differentially expressed (DE) in response to inoculation and between dawn and dusk. Many genes involved in defence related processes were induced more in

response to dawn compared to night inoculations (Chapter.3). Moreover, specific genes previously identified as crucial to the defence response against *B. cinerea* were induced more in response to dawn compared to night inoculations (Chapter 3 – Fig.8). These included genes encoding key defence regulators; ERF1 (Berrocal-Lobo *et al.,* 2002), ERF6 (Moffat *et al.,* 2012) and MYB108 (Mengiste *et al.,* 2003).

Of the 901 DE genes, 99 were identified as transcription factors (TFs). These TFs were significantly enriched for known direct targets of core clock TFs, suggesting a direct link between the circadian clock and the plant defence response pathway against *B. cinerea*. Mining DE TFs for previous links to either the host defence response to *B. cinerea* or/and the central oscillator allowed for the identification of several TFs hypothesized to be linking the clock and defence network (Chapter 3-Table 4).

Not all genes encoding TFs that were DE in response to inoculation in the Windram *et al.,* (2012) study were also DE in this study. The differences in infection times likely play a role in this, as well as this study only capturing the transcriptome at two time points (18 and 22 hours post inoculation (hpi)). Whereas Windram *et al.,* (2012) caught many transient changes in the transcriptome by profiling transcriptome wide changes every two hpi for 48 h.

Jasmonic acid (JA) is a key hormone acting to defend plants against *B. cinerea* colonization (Thomma *et al.*, 1998). It was evident from the transcriptomic profiling in Chapter 3 that the signalling components of the JA signalling pathway were more responsive to inoculations at dawn compared to night. Previous research has established that JA biosynthesis is under circadian regulation (Goodspeed *et al.,* 2012). It has also been shown that JA signalling is under circadian regulation (Appendix 1). The JA receptor, COI1 and a key TF regulating the JA signalling pathway, MYC2, are both regulated by the circadian oscillator; TIME FOR COFFEE (TIC) (Shin *et al.,* 2012). The role of both proteins in the JA signalling pathway has been outlined in Chapter 1. Briefly, COI1 degrades JAZ proteins allowing for the derepression of TFs involved in the JA

signalling pathway and MYC2 is a key regulator of JA related responses and is directly bound by and repressed by JAZ proteins (Pauwels *et al.* (2010); Thines *et al.* (2007)). This direct circadian regulation is likely to contribute to the time-of-day dependent JA signalling responses to *B. cinerea*.

6.1.3 JAZ6 differentially mediates the plant defence response against *B. cinerea* inoculations at dawn compared to night

JAZ proteins are central repressor proteins in the JA signalling pathway and have previously identified as regulators of the defence response against necrotrophic and biotrophic pathogens (Cerrundo *et al.* (2012); de Torres Zabala *et al.* (2015); Leone *et al.* (2014)). JAZ proteins are crucial to the defence response and act upstream in the JA signalling pathway to repress key TFs involved in JA signalling and necrotrophic defences. Such TFs include; MYC2, EIN3, EIL1, bHLH3, 13, 14 and 17, all have been shown to alter resistance to necrotrophic pathogens (Chini *et al.* (2007); Song *et al.* (2013); Zhu *et al.* (2011)). Screening of mutants with altered expression of genes encoding for single or multiple JAZ proteins revealed plants with reduced or constitutive expression of *JAZ6* displayed no differences in resistance against *B. cinerea* whether inoculated at dawn or night (Chapter 4 - Fig.1).

The reduced or constitutive expression of *JAZ6* did not increase resistance against *B. cinerea* after inoculation at dawn but rather reduced the enhanced susceptibility at night (Chapter 4 – Fig.5 and Fig.6). JAZ6 is one of only four JAZ proteins (JAZ5/6/7/8) that contain EAR domain(s) (Kagale *et al.*, 2010). The EAR domain is used by Novel Interactor of JAZ (NINJA) to recruit the co-repressor, Topless (TPL) that then recruits histone deacetylases (HDACs) to repress TFs (Pauwels *et al.,* 2010). The presence of two EAR domains on JAZ6 suggests the protein may repress transcription by directly recruiting TPL without recruiting NINJA. Indeed, JAZ5, JAZ6 and JAZ8 have been shown to directly recruit TPL without NINJA (Causier *et al.* (2012); Shyu *et al.* (2012)). JAZ proteins act to repress TFs in the absence of active JA-Ile, upon the perception of JA-Ile by COI1 JAZ proteins are degraded and TFs are

derepressed, allowing downstream JA responsive genes to be activated (Thines *et al.,* 2007).

6.1.3.1 JAZ6 directly and exclusively bound to TFs involved in the defence response and the circadian clock

Mutants with reduced *MYC2* expression have enhanced resistance to *B. cinerea* and MYC2 has been shown to inhibit the expression of key fungi related defence genes (including *PLANT DEFENSIN 1.2 (PDF1.2)*) (Anderson *et al.* (2004); Lorenzo *et al.* (2004)). MYC2 is therefore a negative regulator of the *B. cinerea* defence response. Given that *jaz6* mutants have increased resistance at night it is more likely JAZ6 is acting at night to repress a positive regulator of the defence response, then in the absence of JAZ6 the positive TF can enhance resistance against *B. cinerea* when inoculated at night. Positive regulators of the defence response include the TFs EIN3/EIL1, which have been shown to be repressive targets of the JAZ proteins (Zhu *et al.,* 2011), however an interaction with JAZ6 was previously unreported until this study.

The yeast-2-hybrid (Y2H) assay confirmed JAZ6 was indeed capable of interacting with EIN3 (Chapter 4 - Fig.12). The Y2H assay employed a library of the majority of TFs discovered in Arabidopsis (Pruneda-Paz *et al.,* 2014). TFs were not only screened against JAZ6, but also JAZ5, JAZ7 and JAZ10. The three other JAZ proteins were screened as negative controls, since plants mutated in the expression of these JAZ encoding genes exhibited no difference to Col-0 in time of day dependent resistance to *B. cinerea*. The aim was therefore to determine what TFs JAZ6 is capable of interacting with that the other JAZ proteins are not, these interactions could explain the conferred circadian regulated resistance to *B. cinerea*.

JAZ6 was found to exclusively interact with a subset of seven TFs, which have previously been implicated in a diverse range of biological processes (Chapter 4 – Fig.12). For example, WOX12 is involved in developmental processes (Constanzo *et al.,* (2014); Haecker *et al.,* (2004)), ZF1 regulates in abiotic

tolerance and plant growth (Kodaira *et al.,* (2011); Sakamoto *et al.,* (2004)), FHY3 is a central regulator of the circadian oscillator (Allen *et al.,* 2006) and, as mentioned, EIN3 is a positive regulator in the defence response against *B. cinerea* (Zhu *et al.,* 2011). The diverse range of TFs bound by JAZ6 reflects the wide range of biological processes JAZ proteins have been shown to mediate. JAZ proteins have previously been shown to bind to the MYC TFs, involved in the defence response (Fernández-Calvo *et al.* 2011), JAM1/2/3 involved in the defence response, anthocyanin accumulation and root growth (Song *et al.,* 2013), ICE1/2 TFs involved in cold tolerance (Hu *et al.,* 2013) and MYB21/24 implicated in male fertility (Song *et al.,* 2011). As research progresses the list of biological processes mediated by the JAZ proteins continually expands.

The interaction with EIN3 is a probable mechanism behind how JAZ6 is differentially regulating the defence pathway in response to dawn compared to night inoculations. This could be further confirmed by confirming JAZ6 and EIN3 bind *in-planta*. The action of EIN3 is outlined in Chapter 1. Briefly, EIN3 is TF that activates the JA/ethylene (ET) mediated defence pathways against necrotrophic pathogens (Song *et al.* (2014); Zhu *et al.* (2011)). EIN3 activates these pathways by binding upstream of genes encoding for TFs (such as ERF1) and instigating their expression (Solano *et al.,* 1998). TFs such as ERF1 then bind to the GCC-box motif found upstream of key defence genes such as *PDF1.2* and *ORA59* (Fig 6.1). This TF interacted with JAZ6 in Y2H and *JAZ6* mutants lost time-of-day dependent resistance to *B. cinerea*. The EIN3 mediated defence response pathway was therefore hypothesized to be the mediated by JAZ6 and hence responsible for the time of day differences observed in resistance to *B. cinerea.* In conjunction with this hypothesis, *ein3-1* mutants lost time of day dependent differences in host resistance to *B. cinerea* (Appendix 7).

Accordingly, the GCC-box motif, which is bound by TFs in the EIN3 mediated pathway, was overrepresented in the upstream sequences of the gene group found to be upregulated more in response to inoculation at dawn compared to night (Fig.6.1). These genes included three plant defensins (*PDF1.2, 1.2b, 1.2c*), a senescence related gene (*SRG3*) and a member of the NAC family (*NAC04*). The

overrepresentation of this motif indicates a streamlining of the defence response following inoculations at dawn. This defence response is likely initiated by the interaction of EIN3 with genes encoding for TFs such as ERF1 or ORA59. The interaction observed between JAZ6 and EIN3 could a time-of-day dependent 'switch' that either activates or inactivates this pathway, allowing the plant to mount an effective or ineffective defence response against *B. cinerea* (Fig 6.1).

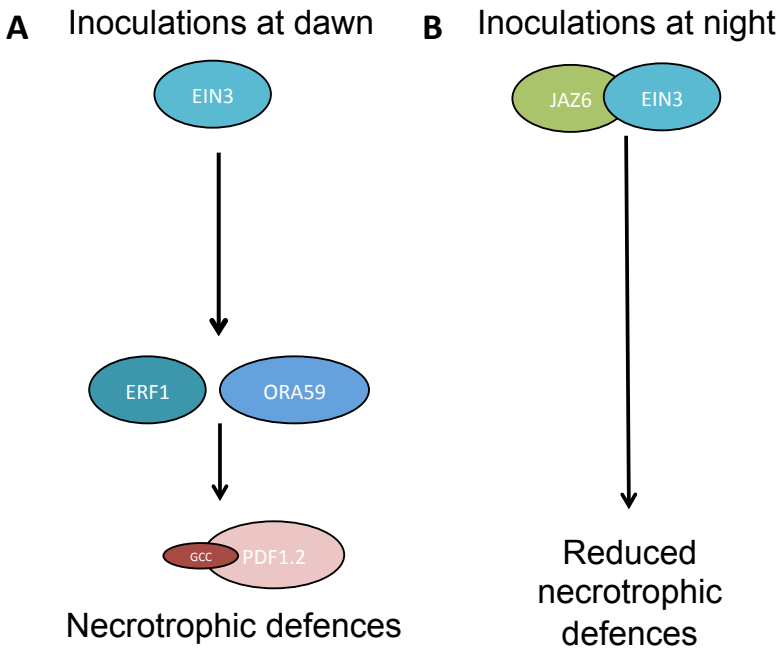


Figure 6.1 – The interaction between JAZ6 and EIN3 is potentially responsible for the time of day dependent resistance to *B. cinerea*. Col-0 plants inoculated at dawn (A) show enhanced resistance to *B. cinerea* inoculation compared to plants inoculated at night (B). The time at which *B. cinerea* begins to elicit host responses after dawn inoculations corresponds to a low level of *JAZ6* expression whereas the time *B. cinerea* begins to elicit host responses after night inoculations corresponds to a high level of *JAZ6* expression. Y2H revealed JAZ6 could interact with EIN3, a positive regulator of the plant defence response against *B. cinerea*. It is possible the JAZ6: EIN3 interaction occurs in response to night inoculations (B) and this explains the enhanced susceptibility of Col-0 plants inoculated at night.

6.1.4 Other JAZ proteins may also differentially mediate the plant defence response against *B. cinerea* at dawn compared to night

Plants altered in the expression of a subset of JAZ genes (JAZ: 5/6/7/10) were screened for time-of-day dependent resistance to *B. cinerea.* If this screen were

widened to include mutants of the remaining eight JAZ genes it is possible other JAZ proteins would also be uncovered as links between the circadian clock and plant defence against *B. cinerea*. For example, JAZ1, JAZ3 and JAZ9 have been shown to bind to EIN3 (Zhu *et al.,* 2011). It is therefore possible mutants of genes encoding for these proteins could be altered in time-of-day dependent resistance to *B. cinerea*. This is especially probable for mutants of JAZ3, given that the diurnal expression of this gene is similar to that of JAZ6 (Chapter 4 – Fig.16).

However, without screening these mutants it is difficult to speculate on the results and roles of individual JAZ proteins given the functional observed within this family. Mutants of these genes were not screened as it was not initially hypothesized EIN3 mediated the time-of-day dependent defences. The selection process for the JAZ mutants that were screened was outlined in Chapter 4. Redundancy among JAZ family members was highlighted in the study by de Torres Zabala *et al.*, (2015) which showed *jaz5/10* mutants displayed greatly reduced resistance to *P. syringae* infection. However, single mutants of either *JAZ5* or *JAZ10* or double mutants of other JAZ genes did not differ in susceptibility levels to *P. syringae* infection.

It is also possible that JAZ6 is the only JAZ protein capable of mediating the circadian defence response to *B. cinerea*. This would not be the first example of a JAZ protein acting independently in the pathogen defence response. For example, a *JAZ10* silenced mutation was shown to increase susceptibility to *B. cinerea* colonization in a light dependent manner (Cerrundo *et al*., 2012). Moreover, a single JAZ7 mutant revealed JAZ7 to independently mediate the defence response against both bacterial and fungal pathogens (Thatcher *et al.,* 2016). This is speculated to be a truly independent action of JAZ7 given that this protein has yet to be shown to dimerize with other JAZ proteins (Chini *et al*. (2009); Shyu *et al.* (2012)).

Other JAZ proteins form homo- and heterodimers with one another (Chini *et al.,* 2009) and with members of other families such as MYC-type and bHLH proteins

(Geerinck *et al.,* 2010). Therefore inferring regulatory relationships and protein functions from plants mutated in the expression of these genes can be unreliable. Mutating the expression of one gene encoding a protein member in such complexes could have inadvertent effects and one protein may not directly instigate the phenotype observed.

In the case of *JAZ6*, it could be that by altering the expression and therefore protein levels of this gene, this alters the protein complex and allows other protein members to bind. Therefore, the phenotype observed may not be due to the altered *JAZ6* expression directly but may be due to other protein members binding in the complex. Given the high level of redundancy among the JAZ proteins it is possible the other protein potentially binding the protein complex is a JAZ protein.

6.1.5 A potential role for DELLA proteins in circadian immunity

Plants must choose whether to allocate resources to growth or defence (Ballaré, 2009). This decision is based on many factors, on the one hand a plant must grow up and out from the canopy to obtain sunlight for photosynthesis, however, plants must also defend against both abiotic and biotic stresses. A low red: far red (R: FR) detected by leaves indicates a shaded canopy and instigates plants to switch to allocating resources to the growth pathways (Ballaré (2009); Cerrudo *et al.* (2012)). The growth and defence pathways are partially regulated by the JA and gibberellin (GA) phytohormones (Robert-Seilaniantz *et al.,* 2011).

DELLA proteins such as, RGA, modulate the GA signalling pathway in a similar manner to the modulation of the JA signalling pathway by the JAZ proteins (Robert-Seilaniantz *et al.,* 2011). DELLA proteins repress GA signalling under times of low GA, when GA is increased, DELLA proteins are degraded via the GA receptor GID (Harberd *et al.,* 2009). DELLA proteins also mediate the cross talk between JA and GA signalling by binding to the JAZ proteins and preventing their repression of JA-responsive TFs such as MYC2/3/4 (Hou *et al.* (2010);

Yang *et al.* (2012)). Hence DELLA proteins are positive mediators of the JA signalling pathway (Navarro *et al.,* 2008). This JAZ and DELLA interaction has been speculated to be the 'switch' allowing plants to either allocate resources into growth or defence (Leone *et al.,* 2014). When GA levels are low DELLA stability is high, and resources are allocated to JA related defences, however, when GA is increased DELLA stability is reduced and resources are reallocated to growth (Leone *et al.,* 2014).

In times of low R: FR plants reduce JA defence related signalling and increase plant growth by increasing the degradation of DELLA proteins (Leone *et al.,* 2014). This removes DELLA proteins from binding to JAZ proteins and allows JAZ proteins to repress the JA signalling pathway (Ballaré (2014); Leone *et al.,* 2014). This repression of the JA pathway coincides with an increase in susceptibility to *B. cinerea* (Cerrudo *et al.,* 2012). However, when *JAZ10* is mutated, light wavelength induced susceptibility to *B. cinerea* is reduced (Cerrudo *et al.,* 2012). This indicates that the relationship between JAZ and DELLA families allows the fine-tuning of the growth and defence pathways.

As well as JAZ6 mutants, several JAZ10 mutants lost circadian mediated defences against *B. cinerea*, *jaz10, jaz5/10* and *jaz5/6/10* mutants all displayed no difference in susceptibility to *B. cinerea* when inoculated at dawn compared to night under cyclic light conditions (Chapter 4). However, a triple *jaz5/7/10* mutant showed a wild-type phenotype, with significantly greater resistance to *B. cinerea* when inoculated at dawn compared to night under cyclic and constant light conditions. This shows a complex web of interactions within the JAZ protein family, the circadian clock and the plant immune response to *B. cinerea*. It is possible that as well as playing a key role in light-wavelength mediated host defences to *B. cinerea* JAZ10 plays a key role in circadian-mediated defences against *B. cinerea*.

Both defence and growth pathways are under circadian regulation. Host defences against *B. cinerea* are under circadian regulation with peak in resistance when inoculated at ZT 0 and peak susceptibility when inoculated at

ZT 18. These time points likely elicit responses in the plant approximately 10 hpi (based on data in Windram *et al.,* 2012) (ZT 10 and ZT 4 respectively). Moreover, it has also been shown growth pathways are under circadian regulation, with maximal growth at ZT 18 and minimum growth occurring at ZT 9 (Nozue *et al.,* 2007). These growth timings coincide with peaks in RGA expression, with maximal gene expression at ZT 9 and lowest expression at ZT 18 (Leone *et al.,* 2014) (Fig.6.2).

It is therefore hypothesized that the circadian regulation of both JAZ and DELLA proteins allows plants to display rhythmicity in both defence and growth levels. JA mediated defence against *B. cinerea* is high at ZT 10, and this coincides with a peak in *RGA* expression levels (a DELLA protein) which occurs at ZT 9 (Fig.6.2) (Leone *et al.,* 2014). Further substantiating DELLA proteins are positive mediators of the defence response. Moreover, at ZT 4 when defence to *B. cinerea* is low, *JAZ6* and *JAZ3* expression levels are highest (Chapter 4 – Fig.16). At ZT 4 levels of *RGA* are also much lower than at ZT 9 (Leone *et al.,* 2014), against highlighting the antagonism between these two pathways (Fig.6.2).

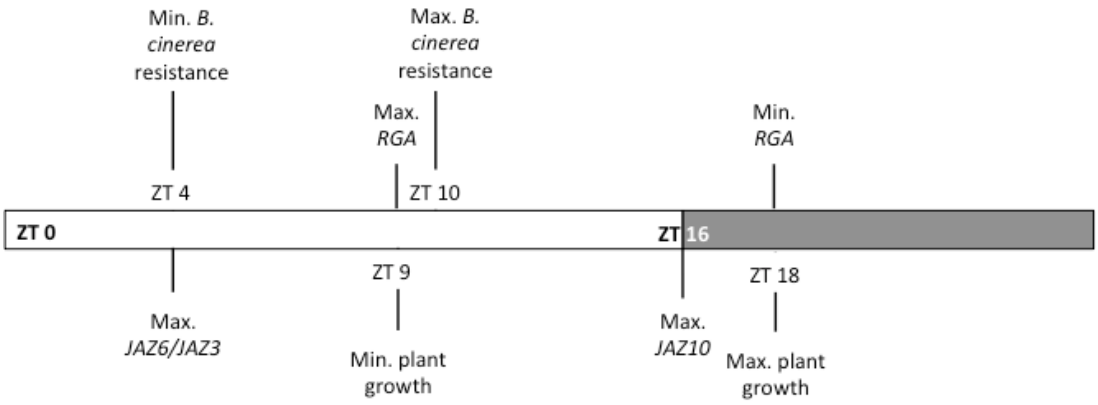


Figure 6.2 – Timing of maximal DELLA expression coincides with timing of maximal resistance to *B. cinerea*. When Arabidopsis leaves are inoculated with *B. cinerea* at ZT 0 (dawn or ZT 18 (night) plants are predicted to display detectable transcriptional changes 10 hpi (based on Windram *et al.,* 2012) so ZT 10 and ZT 4, respectively. The peak in transcriptional responses conferring resistance to *B. cinerea* coincides with a peak in RGA levels. RGA is a DELLA protein and DELLA proteins have been shown to bind to and inhibit the action of JAZ proteins, making DELLA proteins positive mediators of the JA pathway. Information from long-day conditions (16 h light (clear bar): 8 h dark (grey bar))

Transcriptional expression of JAZ10 is at its peak at ZT 16 under long day LD conditions, and levels remain higher throughout the night (ZT 16 – ZT 24) compared to the day (ZT 0 – ZT 16) (Diurnal: Mockler *et al.,* 2007). This peak in expression is likely in anticipation of night, where levels of R: FR are low. Under low R: FR conditions the JAZ10 protein levels are high and act to repress JA signalling (Leone *et al.,* 2014). Increased JAZ10 protein and transcript levels coincide with increased levels plant growth (Fig6.2). The lack of differences between dawn and night susceptibility levels of the *jaz10* mutant to *B. cinerea* was in agreement with previous findings under cyclic lighting (Cerrudo *et al.,* 2012). This, as well as the similar *jaz5/10* phenotype, was possibly influenced by the cyclic light conditions under which the *B. cinerea* assay was conducted.

The *jaz5/7/10* mutant, which was significantly more resistant when inoculated at dawn compared to night under LL or LD conditions, only differs from the *jaz5/10* mutant, which lost circadian mediated immunity, in one gene, JAZ7. The loss of JAZ7 somehow reinstates circadian-mediated immunity against *B. cinerea* in the plant. How this happens needs further investigation.

The *jaz5/6/10* mutant assay which was conducted under both cyclic and constant light conditions consistently showed no difference in resistance levels between inoculations at dawn compared to night independent of lighting conditions. This maybe due to the action of JAZ6 alone which, as discussed, has a prominent role in circadian-mediated immunity.

6.2 Mapping genome wide chromatin accessibility

ATAC-seq uses a simple two-step protocol that, like most protocols, comes with its own set of drawbacks, including mitochondrial reads contaminating data (Buenrostro *et al.,* 2013) and the data analysis tools for ATAC-seq still being in their infancy. The research in Chapter 5 optimized a data analysis pipeline for ATAC-seq data analysis. The inclusion of quality assurance steps allows the researcher to ensure that reads originate from a Tn5 transposase tagmentation event rather than random chromatin shearing. In addition, additional filtering steps allow the exclusion of reads which do not exclusively align to the nuclear

genome and the identification and optional exclusion of reads which are unlikely to be 'true pairs' (defined in Chapter 5).

ATAC-seq has been employed in a wide range of organisms including Drosophila (Davie *et al.,* 2015), humans (Buenrostro *et al.,* (2013); Lara-Astiaso *et al.,* (2014); Prescott *et al.,* (2015)) and 20 other mammals (Villar *et al.,* 2015) to identify genome-wide chromatin accessibility and results have been highly insightful. ATAC-seq has been coupled with transcriptome-wide data to allow researchers to identify the relationship between chromatin accessibility and gene expression. As well as allowing for the identification of TF binding sites with areas of open chromatin throughout the full genome and again being able to relate these back to gene expression. Although ATAC-seq cannot identify the TF bound within the DNA-region, footprinting algorithms such as Wellington (Piper *et al.,* 2013) allow for regions bound by TFs to be analysed for TF binding motifs and from this the likely TF linking the motif can be inferred (Piper *et al.,* 2013).

In relation to this study the integration of ATAC-seq data with the transcriptomic data in Chapter 3, the time-series transcriptomic profiling data and transcriptional networks (from Windram *et al.,* 2012) would be greatly advantageous. This would enable strong predictions about co-regulation of gene groups, which TFs are regulating these gene groups and the dynamics of this regulation at different times of day. However, numerous obstacles prevented the generation of ATAC-seq data in Chapter 5, these included a high proportion of reads aligning to non-nuclear organelles as well as the chromatin conformation being disrupted upon Tn5 transposase treatment.

ATAC-seq data on plant cells is limited, with only the data in Chapter 5 and a dataset published in August 2016 (Wilkins *et al.,* 2016) available. Wilkins *et al.,* (2016) carried out ATAC-seq and transcriptome-wide time-series profiling on *Oryza sativa* (rice) samples following numerous abiotic stresses. Rice ATAC-seq data was combined with transcriptomic profiling as well as previous knowledge

of *cis*-regulatory motifs and this enabled the identification of a high-resolution gene regulatory network in rice.

However, similar to the data in Chapter 5, Wilkins *et al.,* (2016) had low nuclear genome coverage within each sample due to contamination from reads aligning to chloroplast and/or mitochondrial genomes (~70% and ~15%). This low coverage prevented the identification of TF footprints within individual samples and samples were pooled. The proposed an optimized protocol in Chapter 5 outlines potential ways to reduce contamination from reads that align to the chloroplast and mitochondrial genome and hence will improve data resolution in future plant ATAC-seq studies.

6.3 Evolutionary advantage of time-of-day dependent regulation of defences

Why the host circadian clock regulates defences against *B. cinerea* is open to many hypotheses. One hypothesis is that the host anticipates inoculation from *B. cinerea* spores is most likely at dawn, the host clock therefore not only upregulates genes related *B. cinerea* defence in anticipation for this, but it also gates these genes, allowing for them to respond more rapidly to dawn inoculations compared to inoculations at night.

This is hypothesis is likely as previous studies have confirmed spore levels of several fungi are indeed higher at dawn. Circadian and diurnal rhythms of sporulation have been observed in a wide variety of fungi, and have different phases throughout the day (Ingold, 1971). This is to be expected as the factors that influence spore dispersal or survival: temperature, humidity and wind, generally show daily periodicity. These spore release rhythms persist in some species even in constant conditions in the laboratory (Canessa *et al.* (2013); Ingold (1971); Pittendrigh *et al.* (1959)) indicating an endogenous circadian oscillator. Indeed, Hevia *et al.,* (2015) showed *B. cinerea* B05.10 possesses *BcFRQ1,* a functional ortholog of the *Neurospora crassa* circadian clock gene *FREQUENCY* (*FRQ*), giving the fungus rhythmicity. These rhythms result in daily

cycles of spore abundance in the air, with studies showing spore abundance peaking between morning and early afternoon (Hartill, 1980). *B. cinerea* conidia are released between morning and early afternoon due to the increase in light, temperature and reduction in humidity (Williamson *et al.,* 2007).

This hypothesis is not in agreement with the previously discussed results of the Hevia *et al.,* (2015) study which led authors to conclude the fungal clock was dominating the infection outcome between *B. cinerea* and Arabidopsis. Hevia *et al.,* (2015) stated *B. cinerea* BO5.10 is more virulent when it perceives the time as night and the fungal clock and not the plant clock dominates the outcome of infections. However, due to the rapidly evolving nature of the plant and pathogen interactions, if the fungus were indeed more virulent in the evening then plant defences would have most likely evolved to account for this and have increased nighttime defences to limit fungal damage on a night. Nevertheless results in Chapter 3, as well as work by Shin *et al.,* (2012), has indicated host defences are more responsive when inoculations occur at dawn compared to at night, likely due to circadian gating of the JA pathway. Moreover, the differences observed in Ingle *et al.* (2015) between inoculations that occur at dawn and those that occur at night were all observed independent of the fungal clock and were entirely driven by the plant clock. The *B. cinerea* used in all inoculations was cultured for 14-days under constant temperature and light conditions so the fungal clock received no entrainment hence could not have dominated this interaction. Furthermore, when the plant clock was mutated the differences observed between inoculations that occurred at dawn and those that occurred at night were abolished .

6.4 Agricultural impact

Goodspeed *et al.,* (2012) discovered Arabidopsis plants show circadian regulated defences against herbivory by *Trichoplusia ni* (cabbage loopers). Further to this, it was shown circadian regulation of defences exists in economically important crops such as lettuce, spinach, zucchini, sweet potatoes, carrots and blueberries (Goodspeed *et al.,* 2013). Experiments revealed post-

harvest crop tissue incubated under light: dark (LD) cycles reflecting the natural host and herbivore environment suffered reduced losses due to herbivory (Goodspeed *et al.,* 2013).

The circadian clock also plays a vital role in plant defences against *Botrytis cinerea* (Hevia *et al*. (2015); this study). It is therefore possible that by keeping plants under LD cycles indicative of environmental growth conditions during post-harvest transport and storage the plants will maintain a higher level of resistance to herbivory and also *B. cinerea* infection. This is vital given most economic crop damage caused by *B. cinerea* occurs post-harvest (Coley-Smith *et al*. (1980); Williamson *et al*. (2007)). However, the role the plant clock plays in the *B. cinerea* defence response needs to be further investigated to determine this.

JAZ proteins are highly conserved and have been uncovered in a diverse range of economically significant crops including; maize (Zhou *et al.,* 2015), rice (Ye *et al.,* 2009), grape (Zhang *et al.,* 2012), tobacco and tomato (Ishiga *et al.,* 2013). JAZ proteins in these crops have also been shown to mediate pathogen defences (Ishiga *et al.,* 2013). It is therefore possible that JAZ6 or another closely related JAZ family member is acting to regulate the circadian defence response in plants other than Arabidopsis. Indeed, two economically important crops, chickpea and *Brassica rapa* contain *CaJAZ6* and *BrTIFY11b,* which share a high level of sequence similarity to *JAZ6* (also referred to as *TIFY11b*) (Saha *et al.* (2016); Singh *et al.* 2015)). This hypothesis should be further investigated in economically important crops species.

In this study it was found in Arabidopsis plants essentially mount an effective defence response when inoculated at dawn but an ineffective defence when inoculated at night. This comparison has revealed potential key regulators required to mount an effective defence response. Investigating this in economically important crops could further elucidate a plants natural defence response network to *B. cinerea*. Techniques such as genome editing (GE), genetic modification (GM) or marker-assisted breeding could introduce or

enhance this natural defence response to create plants with a constitutively high level of defence against *B. cinerea*. For example, to create Arabidopsis plants with constitutively high levels of resistance against *B. cinerea JAZ6* should be altered in expression.

Altering genes in economically important crops can often introduce undesirable traits. For example, if *MYC2* was reduced in expression with the aim of increasing Arabidopsis tolerance to *B. cinerea* infection, the plant would be more resistant to *B. cinerea* but it would also become much more susceptible to herbivory (Song *et al.* (2014); Zhu *et al.* (2011)). It is essential these impacts be considered, as when plants are in a natural environment they are exposed to myriad of abiotic and biotic stresses. Altering the expression of a JAZ gene is less likely to have such an inadvertent impact as JAZ proteins are numerous and have a high level of familial functional redundancy (Pauwels and Goossens, 2011).

6.5 Future work

All future work is summarized in Figure 6.3 and expanded below.

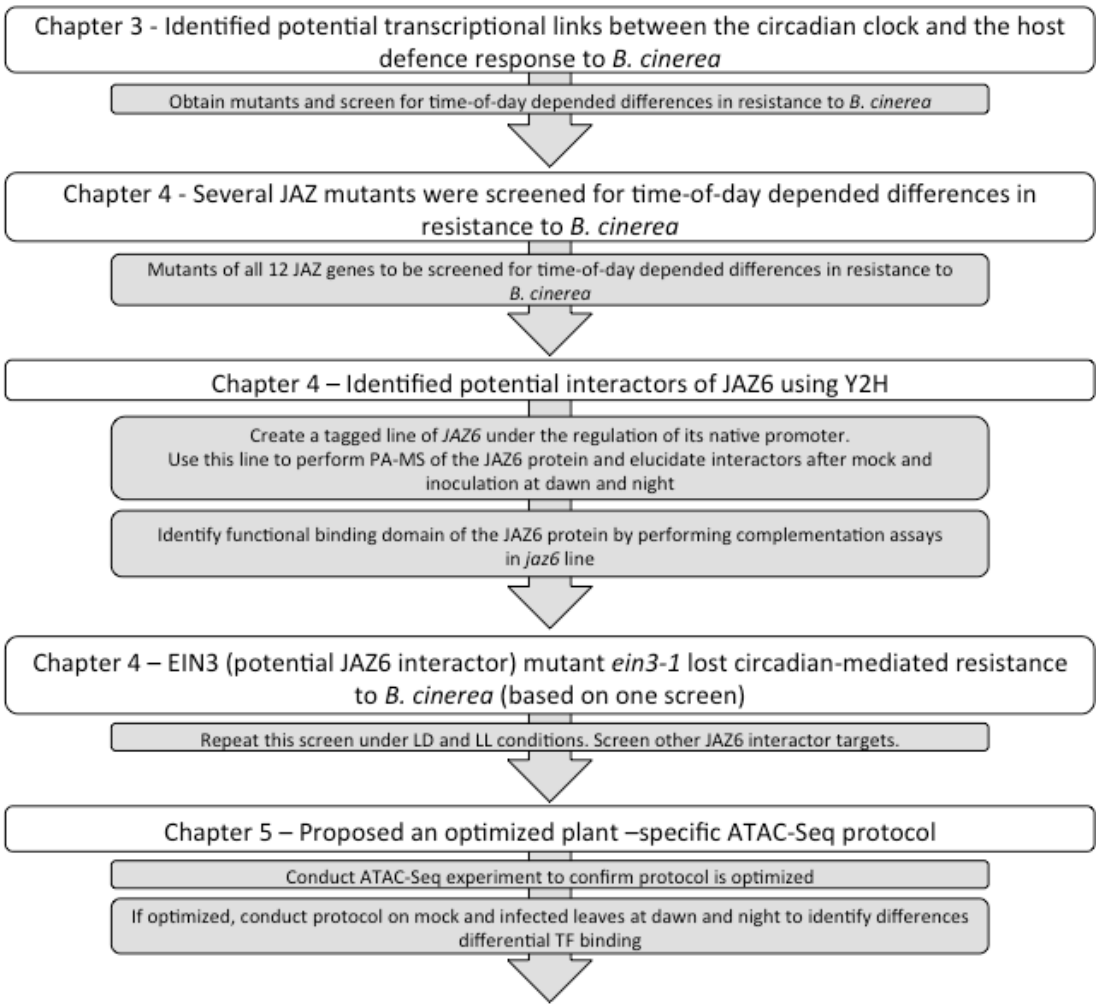


Figure 6.3 – Summary of future work. yeast-2-hybrid (Y2H) pull-down assays followed by mass spectrometry (PA-MS) constant light (LL) cyclic light:dark conditions (LD) transcription factor (TF)

Arabidopsis mutants of genes identified in Chapter 3 as potential transcriptional links between the circadian clock and the host defence response to *B. cinerea* (such as *MYB108*) should be generated or obtained and subsequently screened for time-of-day depended differences in resistance to *B. cinerea.* It is likely these TFs are crucial to the defence response given that many were DE 18 or 22 hpi at ZT 0 or/and ZT 18 in this study as well as DE between 2-48 hpi at ZT 6 (Windram *et al.,* 2012).

Chapter 4 identified JAZ6 as a key regulator of the circadian regulated defence response against *B. cinerea* by screening mutants with reduced JAZ6 expression. This was performed for several other JAZ mutants but not all twelve JAZ family members.

The interactions in Chapter 4 were obtained from a Y2H assay; again this methodology comes with its own set of limitations, the main issue is reliability of results. Y2H screens are an initial indication as to what is capable of binding and are should always be validated using *in-planta* techniques such as pull-down assays followed by mass spectrometry (PA-MS), bimolecular fluorescence complementation (BIFC) and/or co-immunoprecipitation (Co-IP). Future work should focus on elucidating how JAZ6 is influencing the defence response differently in response to dawn compared to night inoculations. A tagged line of *JAZ6* under the regulation of its native promoter should be created to determine this. A tagged line would allow JAZ6 protein accumulation to be tracked throughout the 24-hour day and protein expression could then be correlated to transcript accumulation. Moreover, this line could be inoculated and mock inoculated at dawn and night and this tissue could then be used for PA-MS of the JAZ6 protein. This would elucidate the role of JAZ6 in the defence response, as it would indicate what TFs JAZ6 was binding and how these interactions differ between dawn and night inoculations.

The binding domain JAZ6 uses to interact with proteins and regulate the circadian-mediated defences against *B. cinerea* should also be elucidated. Functional complementation assays into the *jaz6* mutant line would allow this. Constructs of the JAZ6 gene with and without the region coding for the EAR domain(s) could be expressed in the *jaz6* line. These lines could then be screened for resistance to *B. cinerea* at dawn and night to determine which EAR domain(s) is responsible for interactions, which lead to circadian-mediated immunity to *B. cinerea*.

Chapter 4 also identified a protein interactor of JAZ6, which has a known role in *B. cinerea* defence, EIN3. A mutant with reduced expression of the *EIN3*

transcript (*ein3-1*) mutant lost any differences in resistance to *B. cinerea* when inoculated at dawn compared to night. However, this was based on one screen only and is contradictory to previous research. This requires repeating.

Chapter 5 proposed a protocol optimization based on an informed set of molecular and bioinformatics analyses and comparisons for plant-based ATAC-seq studies. Future work should focus on affirming the optimization of this protocol. Work could then focus on employing this protocol to investigate chromatin accessibility and differential TF binding between mock and inoculated plants at the times specified in Chapter 5 to be when the plant is likely exhibiting a differential immune response. This data would be combined with transcriptomic profiling time-series data (Windram *et al.,* 2012) data to enable informed predictions of genome wide regulation in response to *B. cinerea* infection at different times of day.

6.6 Conclusions

This study elucidated numerous potential mechanisms linking the circadian clock to the host defence response against *B. cinerea*. Although the circadian clock's regulation of the stomatal aperture is likely important in regulating defence against non-necrotrophic pathogens, it was revealed *B. cinerea* does not preferentially enter via stomata. Transcriptomic profiling of plants inoculated at subjective dawn or night revealed the expression of a subset of JA responsive genes was upregulated more in response to dawn compared to night inoculations. Moreover, inoculating mutants of the JA signalling pathway at subjective dawn or subjective night with *B. cinerea* revealed JAZ6 to be mediating the link between the circadian clock and the plant defence response against *B. cinerea*. With JAZ6 serving as a link between the two pathways, a protein-binding assay revealed JAZ6 could bind a central regulator of the host defence response against *B. cinerea* (EIN3) and a TF found to be regulating the central oscillator (FHY3).

Moreover, this study also optimized a protocol to implement ATAC-seq on plant tissue, which will allow for the identification of genome-wide chromatin accessibility and *cis*-regulatory regions. As well as this, an analysis pipeline for ATAC-seq data analysis was outlined.

7: Appendices

Appendix 1: Genes involved in JA biosynthesis, metabolisis or perception/signalling are significantly enriched for circadian clock binding compared to the rest of the genome

Genes sourced from Wasterneck *et al.,* (2013) review. Direct targets of core circadian clock transcription factors (TOC1 (Huang *et al.* 2012), PRR5 (Nakamichi *et al.* 2012), PRR7 (Liu *et al.* 2013a) and CCA1(Kamioka *et al*., 2016) identified using ChIP-seq.

JA process	AGI	Gene name	Clock TF direct targets			
			TOC1	PRR5	PRR7	CCA1
biosynthesis	AT2G44810	DAD1	-	-	-	-
biosynthesis	AT4G18960	AG	-	-	-	-
biosynthesis	AT1G05800	DGL	-	-	-	-
biosynthesis	AT1G06800	PLA1γ1	-	-	-	-
biosynthesis	AT3G45140	LOX2	yes	-	-	-
biosynthesis	AT1G17420	LOX3	yes	-	-	-
biosynthesis	AT1G72520	LOX4	yes	-	-	-
biosynthesis	AT1G67560	LOX6	-	-	-	-
biosynthesis	AT3G15030	TPC4	-	-	-	-
biosynthesis	AT5G42650	AOS	-	-	-	-
biosynthesis	AT3G25760	AOC1	-	-	-	-
biosynthesis	AT3G25770	AOC2	-	-	-	yes
biosynthesis	AT3G25780	AOC3	-	-	-	-
biosynthesis	AT1G13280	AOC4	-	-	-	-
biosynthesis	AT2G06050	OPR3	-	-	-	yes
biosynthesis	AT4G15440	HPL	-	-	-	-
metabolisis	AT5G06950	TGA2	-	-	-	-
metabolisis	AT5G06960	TGA5	-	-	-	-
metabolisis	AT3G12250	TGA6	-	-	-	-
metabolisis	AT1G07530	SCL14	-	-	-	-
metabolisis	AT3G48520	CYP94B3	-	-	-	-
metabolisis	AT5G07010	AtST2a	-	-	-	yes
metabolisis	AT1G04220	LCF	-	-	-	-
perception/signalling	AT5G63970	RGLG3	-	-	-	-
perception/signalling	AT1G32640	MYC2	-	-	-	-
perception/signalling	AT5G46760	MYC3	-	-	-	-
perception/signalling	AT4G17880	MYC4	-	-	-	-
perception/signalling	AT5G41315	GL3	-	-	-	-
perception/signalling	AT1G63650	EGL3	-	-	-	-
perception/signalling	AT4G09820	TT8	-	-	-	-
perception/signalling	AT1G02300	PAP	-	-	-	-
perception/signalling	AT3G27920	GL1	-	-	-	-
perception/signalling	AT3G27810	MYB21	-	-	-	-
perception/signalling	AT5G40350	MYB24	-	-	-	-
perception/signalling	AT3G20770	EIN3	-	-	-	-
perception/signalling	AT2G27050	EIL	-	-	-	-
perception/signalling	AT1G14920	GAI	-	-	-	-
perception/signalling	AT2G01570	RGA	-	yes	-	-
perception/signalling	AT1G66350	RGL1	-	-	-	-
perception/signalling	AT5G63110	HDA6	-	-	-	-
perception/signalling	AT4G38130	HDA19	-	-	-	-
perception/signalling	AT5G24770	VSP2	-	-	-	-
perception/signalling	AT3G20840	PLT1	-	-	-	-
perception/signalling	AT1G51190	PLT2	-	-	-	-
perception/signalling	AT2G39940	COI1	-	-	-	-
perception/signalling	AT4G28910	NINJA	-	-	-	-
perception/signalling	AT1G15750	TPL	-	yes	-	-
perception/signalling	AT2G46370	JAR1	-	-	-	-
perception/signalling	AT1G19180	JAZ1	yes	-	-	yes
perception/signalling	AT1G74950	JAZ2	-	-	-	-
perception/signalling	AT3G17860	JAZ3	-	-	-	-

perception/signalling	AT1G48500	JAZ4	-	-	-	-
perception/signalling	AT1G17380	JAZ5	yes	-	-	-
perception/signalling	AT1G72450	JAZ6	-	yes	yes	-
perception/signalling	AT2G34600	JAZ7	-	-	-	-
perception/signalling	AT1G30135	JAZ8	-	-	-	-
perception/signalling	AT1G70700	JAZ9	yes	yes	-	-
perception/signalling	AT5G13220	JAZ10	-	-	-	-
perception/signalling	AT3G43440	JAZ11	-	-	-	-
perception/signalling	AT5G20900	JAZ12	-	-	-	-

Appendix 2: 510 Gene CSI Network

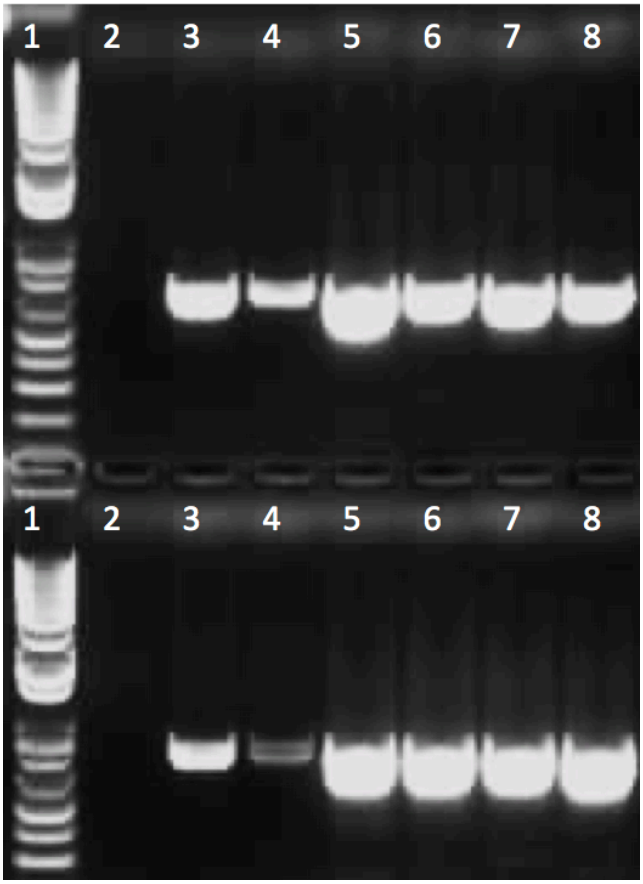
AT1G01060	AT1G71030	AT2G42300	AT3G57390	AT5G01310
AT1G01520	AT1G71260	AT2G42380	AT3G57800	AT5G02470
AT1G01720	AT1G71520	AT2G43000	AT3G58710	AT5G02840
AT1G02170	AT1G72030	AT2G44430	AT3G59060	AT5G03150
AT1G02220	AT1G72050	AT2G45050	AT3G60030	AT5G03220
AT1G02680	AT1G72310	AT2G45100	AT3G60530	AT5G03780
AT1G03650	AT1G72450	AT2G45420	AT3G61250	AT5G04110
AT1G03970	AT1G72650	AT2G45460	AT3G61310	AT5G04340
AT1G04100	AT1G72740	AT2G45660	AT3G61420	AT5G04760
AT1G04370	AT1G73410	AT2G46260	AT3G62420	AT5G05410
AT1G04850	AT1G74080	AT2G46510	AT3G62690	AT5G05610
AT1G05380	AT1G74840	AT2G46590	AT3G63030	AT5G05790
AT1G05420	AT1G75390	AT2G47190	AT4G00050	AT5G06550
AT1G05690	AT1G75410	AT2G47810	AT4G00120	AT5G06770
AT1G05710	AT1G75490	AT2G48160	AT4G00220	AT5G06950
AT1G05830	AT1G75510	AT3G01080	AT4G00260	AT5G06960
AT1G06040	AT1G76110	AT3G01140	AT4G00270	AT5G07060
AT1G06160	AT1G76500	AT3G01460	AT4G00730	AT5G07580
AT1G06170	AT1G76580	AT3G01970	AT4G00760	AT5G08330
AT1G06180	AT1G76710	AT3G02380	AT4G00850	AT5G08430
AT1G08320	AT1G76880	AT3G02550	AT4G00940	AT5G08630
AT1G08540	AT1G76890	AT3G02860	AT4G00990	AT5G08790
AT1G08620	AT1G77450	AT3G03760	AT4G01120	AT5G09330
AT1G09030	AT1G78080	AT3G04060	AT4G01550	AT5G09460
AT1G09060	AT1G78930	AT3G04070	AT4G02640	AT5G09750
AT1G09710	AT1G79000	AT3G04590	AT4G03160	AT5G10510
AT1G09770	AT1G79150	AT3G04670	AT4G03250	AT5G11260
AT1G09950	AT1G79220	AT3G04730	AT4G04404	AT5G11270
AT1G10120	AT1G79430	AT3G04740	AT4G04890	AT5G11590
AT1G10320	AT1G79700	AT3G05200	AT4G05100	AT5G12440
AT1G10450	AT1G80840	AT3G05545	AT4G05170	AT5G12840
AT1G10586	AT2G01810	AT3G05690	AT4G06746	AT5G13080
AT1G12440	AT2G02450	AT3G05760	AT4G09180	AT5G13180
AT1G13260	AT2G02470	AT3G06400	AT4G10350	AT5G13220
AT1G13300	AT2G02820	AT3G06490	AT4G10920	AT5G13240
AT1G13450	AT2G03340	AT3G07260	AT4G11080	AT5G13330
AT1G14410	AT2G03710	AT3G10330	AT4G11680	AT5G14260
AT1G14510	AT2G04740	AT3G10500	AT4G12080	AT5G14280
AT1G14920	AT2G04880	AT3G10820	AT4G13040	AT5G15030
AT1G15340	AT2G05330	AT3G11090	AT4G13480	AT5G15160
AT1G16690	AT2G13370	AT3G11200	AT4G13640	AT5G15850
AT1G18710	AT2G17040	AT3G11280	AT4G13980	AT5G17430
AT1G19180	AT2G17560	AT3G11580	AT4G14605	AT5G17690

AT1G19860	AT2G17950	AT3G12210	AT4G14720	AT5G18230
AT1G20693	AT2G18160	AT3G12270	AT4G14920	AT5G18550
AT1G21200	AT2G18500	AT3G12480	AT4G15090	AT5G18830
AT1G22070	AT2G19520	AT3G13000	AT4G16150	AT5G20510
AT1G22590	AT2G19810	AT3G13040	AT4G16420	AT5G20900
AT1G22985	AT2G20110	AT3G13350	AT4G17050	AT5G22760
AT1G24610	AT2G21060	AT3G13960	AT4G17460	AT5G23280
AT1G25440	AT2G21230	AT3G15210	AT4G17500	AT5G24120
AT1G25550	AT2G21320	AT3G15270	AT4G17600	AT5G24800
AT1G25580	AT2G21530	AT3G15500	AT4G17810	AT5G25470
AT1G26260	AT2G22300	AT3G16280	AT4G18170	AT5G25830
AT1G26310	AT2G22430	AT3G16350	AT4G18650	AT5G27620
AT1G27000	AT2G22540	AT3G16940	AT4G19985	AT5G29000
AT1G27220	AT2G22800	AT3G18380	AT4G20400	AT5G33210
AT1G27730	AT2G23320	AT3G18990	AT4G20810	AT5G35770
AT1G30135	AT2G23740	AT3G19580	AT4G21430	AT5G37260
AT1G31150	AT2G24650	AT3G20310	AT4G21750	AT5G38860
AT1G31320	AT2G25000	AT3G20670	AT4G22070	AT5G39610
AT1G31640	AT2G25820	AT3G20770	AT4G22140	AT5G39760
AT1G32070	AT2G27230	AT3G20800	AT4G22360	AT5G41090
AT1G32150	AT2G27990	AT3G21150	AT4G22745	AT5G41370
AT1G32540	AT2G28200	AT3G21890	AT4G23810	AT5G41920
AT1G32700	AT2G28290	AT3G23030	AT4G25210	AT5G42290
AT1G32770	AT2G28500	AT3G23130	AT4G25380	AT5G42520
AT1G34370	AT2G28710	AT3G23210	AT4G26500	AT5G43270
AT1G35460	AT2G29580	AT3G23220	AT4G26640	AT5G43700
AT1G43860	AT2G30470	AT3G23230	AT4G26930	AT5G43840
AT1G46480	AT2G31230	AT3G23240	AT4G27410	AT5G47230
AT1G48000	AT2G31280	AT3G23250	AT4G27900	AT5G47370
AT1G48310	AT2G32645	AT3G24050	AT4G28640	AT5G47640
AT1G49130	AT2G32700	AT3G24120	AT4G28790	AT5G49200
AT1G49475	AT2G32950	AT3G24820	AT4G28811	AT5G49300
AT1G49480	AT2G33480	AT3G25710	AT4G30080	AT5G49450
AT1G49720	AT2G33610	AT3G27940	AT4G30180	AT5G49520
AT1G50640	AT2G33620	AT3G28210	AT4G31615	AT5G50010
AT1G51070	AT2G33810	AT3G28730	AT4G31650	AT5G50320
AT1G52520	AT2G34600	AT3G28910	AT4G32570	AT5G51990
AT1G53320	AT2G34710	AT3G44750	AT4G32800	AT5G52510
AT1G55460	AT2G34820	AT3G46620	AT4G34530	AT5G54180
AT1G55750	AT2G35700	AT3G47610	AT4G35040	AT5G55580
AT1G59940	AT2G35940	AT3G48600	AT4G35540	AT5G56780
AT1G61110	AT2G36560	AT3G49930	AT4G35550	AT5G56960
AT1G61960	AT2G36740	AT3G50330	AT4G35580	AT5G57180
AT1G61970	AT2G36990	AT3G51910	AT4G36240	AT5G57660
AT1G62300	AT2G37430	AT3G51950	AT4G36590	AT5G58900

AT1G64530	AT2G38130	AT3G52540	AT4G36620	AT5G59430
AT1G64860	AT2G38470	AT3G52800	AT4G36900	AT5G59570
AT1G66370	AT2G38950	AT3G53370	AT4G36990	AT5G60480
AT1G67030	AT2G39020	AT3G53600	AT4G37610	AT5G60970
AT1G68480	AT2G39250	AT3G53920	AT4G37750	AT5G61420
AT1G68520	AT2G39900	AT3G54220	AT4G37790	AT5G62000
AT1G68840	AT2G40140	AT3G54390	AT4G38160	AT5G62020
AT1G69010	AT2G40620	AT3G54620	AT4G38170	AT5G62430
AT1G69490	AT2G40670	AT3G55980	AT4G38180	AT5G62470
AT1G69690	AT2G41630	AT3G56400	AT4G38620	AT5G63080
AT1G69810	AT2G41710	AT3G56530	AT4G38900	AT5G63160
AT1G70060	AT2G41940	AT3G57300	AT4G40060	AT5G63900
AT5G64340	AT5G65310	AT5G65670	AT5G66270	AT5G67110
AT5G64810	AT5G65490	AT5G65910	AT5G66320	AT5G67580

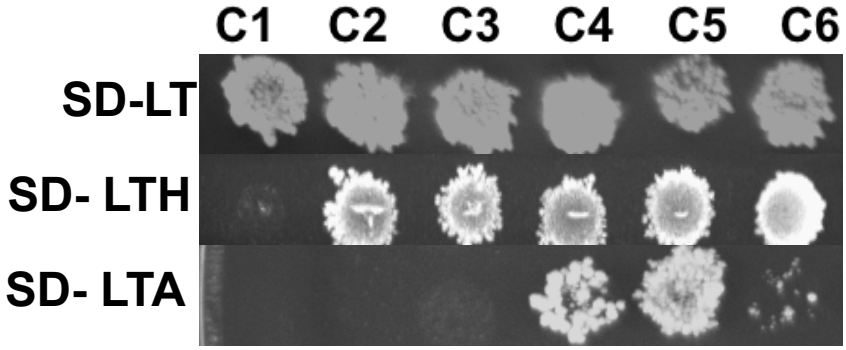
Appendix 3: *JAZ6-OX* mutant line contains the correct overexpression vector

PCR of Col-0 and *JAZ6-OX* mutant plants 1-12 (Lanes 3-8) using primers specific to the *35S* promoter region and *JAZ6* hence why no band is seen in the Col-0 lanes (Lanes 2). Band size expected between *35S* and *JAZ6* is 953 bp. All *JAZ6-OX* plants displayed this band as can be seen below. Ladder employed 1 Kb Plus DNA Ladder (Life Technologies).



Appendix 4: Selective media controls employed throughout Y2H assay

Controls to indicate media was lacking in the correct components was employed throughout the Y2H assay. Details on controls (C) one to five can be found in Dreze *et al.,* (2010).



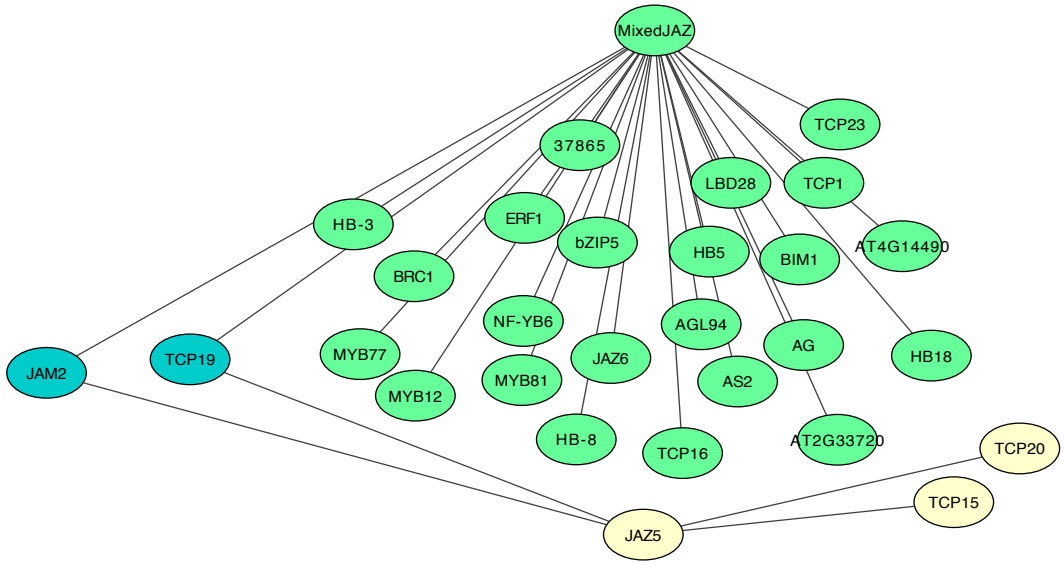
Appendix 5: Y2H AD TF library

TF library screened against JAZ6 DB and Mixed JAZ pool DBs included all those outlined in Pruneda-Paz *et al.,* (2014) minus those outlined below which were not successfully be cloned:

- AT5G49520
- AT5G66770
- AT4G31620
- AT3G20770
- AT4G00730

Appendix 6: Individual JAZ5 DB Y2H screen against 768 TFs from AD library

JAZ5 DB was individually screened against 768 proteins in the TF library. 4 proteins in this group repeatedly interacted with JAZ5. 2 of these proteins had also been shown to bind with the mixed JAZ pool (turquoise) and the JAZ5 DB exclusively bound 2 proteins, which did not interact with the pooled JAZs (lemon). Of the 768 proteins screened, 25 had been shown to interact with the mixed JAZ pool only and not the JAZ5 DB alone (green).



Appendix 7: Extra information on TFs found to interact with JAZ proteins.

Information includes AGI number, Gene name, interactions recorded within Chapter 4, expression information (ePlant (https://bar.utoronto.ca/eplant/) (Fucile *et al.,* 2011)) (LE – leaf 7 expression), any previous phenotype recorded when infected with *B. cinerea* (WL – white light) and any previous Y2H results (BioGrid). Within LE column – lowly is defined as low expression, below 0.5 on a Log2 ratio scale. Y - yes.

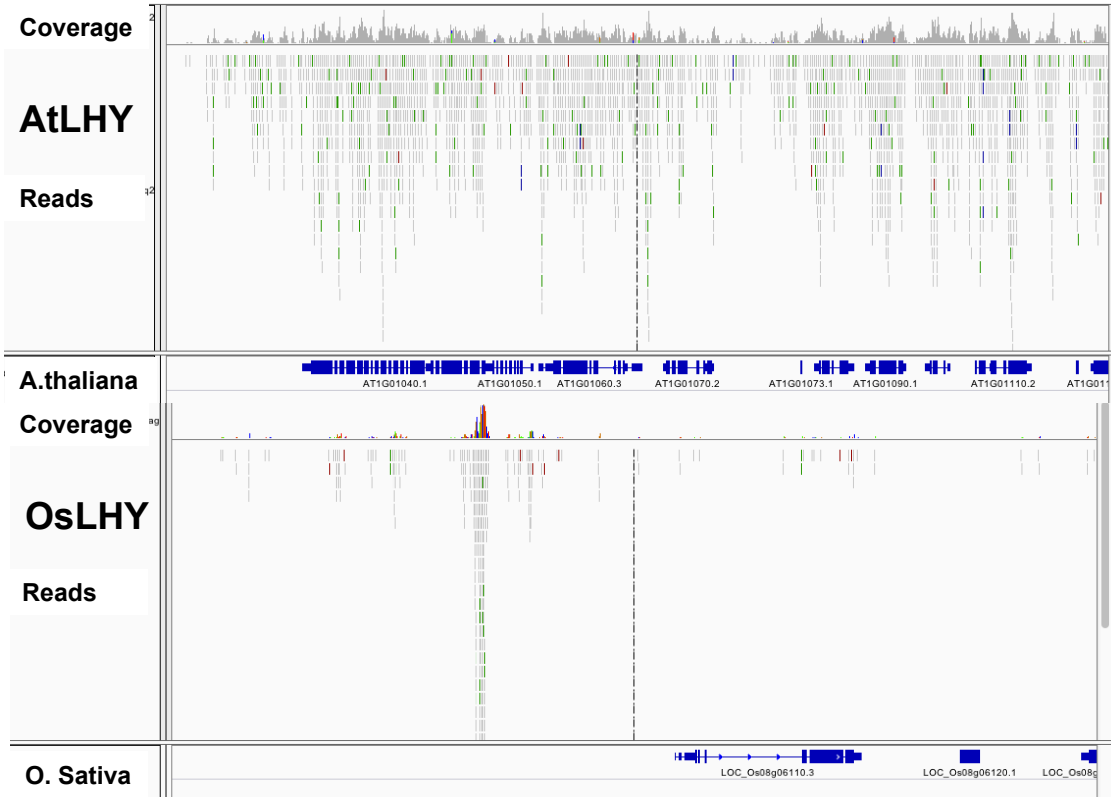
AGI	Target list	Y2H - JAZs				LE	Previous phenotype with Botrytis	Previous Y2H results (BioGrid)			
		5	6	7	10			JAZs	MYCs	TPLs	Clock proteins
AT5G46830	**NIG1**	y	-	-	y	y	-	1/2/6/8/10/11	-	-	-
AT1G24260	**SEPELLATA 3**	y	-	-	y	lowly	-	-	-	-	-
AT5G08130	**BIM1**	y	-	-	y	y	-	-	-	-	-
AT3G54990	**SMZ**	-	-	-	y	lowly	-	-	-	-	-
AT4G32880	**HB-8**	-	-	-	y	lowly	-	-	-	-	-
AT5G23280	**TCP7**	y	y	-	y	-	-	-	-	-	-
AT5G08330	**TCP21**	y	y	-	y	y	-	-	-	-	TOC1
AT1G35560	**TCP23**	y	y	-	y	y	-	-	-	TPR3	-
AT5G51910	**TCP19**	y	y	-	y	y	-	-	-	-	-
AT2G33720	**AT2G33720**	y	-	y	y	y	-	-	-	-	-
AT4G18960	**AG**	y	-	y	y	lowly	-	-	-	-	-
AT3G18550	**BRC1**	y	-	y	y	-	-	-	-	-	-
AT3G50510	**LBD28**	y	-	y	y	-	-	-	-	-	-
AT3G12910	**NAC UK**	-	-	y	y	lowly		-	-	-	-
AT1G65620	**AS2**	-	-	y	y	lowly	-	-	-	-	-
AT1G69690	**TCP15**	y	y	y	y	y	-	-	-	-	PRR5
AT3G50060	**MYB77**	y	y	y	y	y	-	-	-	TPL/TPR1	-
AT2G45680	**TCP9**	y	y	y	y	y	-	-	-	-	-
AT1G67260	**TCP1**	y	y	y	y	-	-	-	-	-	-
AT1G58100	**TCP8**	y	y	y	y	y	-	-	-	-	-
AT4G17880	**MYC4**	y	y	y	y	y	myc2/3/4 more susc. In WL (Chico, 2014)	all	2, 3 and 4	-	-
AT5G46760	**MYC3**	y	y	y	y	y				-	HY5
AT1G01260	**JAM2**	y	y	y	y	y	-	1/2/3/4/5/6/8/9/10/11	-	-	-

AT2G26960	**MYB81**	y	y	y	y	y	-	-	-	-	-
AT1G69540	**AGL94**	y	y	y	y	guard cell	-	-	-	-	-
AT3G45150	**TCP16**	y	y	y	y	-	-	-	-	TPR3	-
AT1G32640	**MYC2**	y	y	y	y	y	myc2 - more res. (Lorenzo, 2004)	all	2, 3 and 4	-	TIC
AT3G23240	**ERF1**	y	-	-	-	y	ERF1-OX more res. (Berrocal -Lobo, 2002)	-	-	-	-
AT5G47670	**NF-YB6**	y	-	-	-	lowly	-	-	-	-	-
AT4G14490	**AT4G14490**	y	-	-	-	y	-	-	-	-	-
AT1G70920	**HB18**	y	-	-	-	lowly	-	-	-	-	-
AT2G47460	**MYB12**	-	-	-	-	lowly	-	-	-	-	-
AT5G09460	**AT5G09460**	-	-	-	-	y	-	-	-	-	-
AT1G31760	**SWIB**	-	-	-	-	lowly	-	-	-	-	-
AT5G65310	**HB5**	-	-	-	-	y	-	-	-	-	-
AT3G02150	**PTF1**	y	y	-	-	y	-	-	-	-	-
AT5G56840	**myb like**	-	y	-	-	-	-	-	-	-	-
AT4G23860	**AT4G23860**	-	y	-	-	y	-	-	-	-	-
AT5G17810	**WOX12**	-	y	-	-	-	-	-	-	-	-
AT1G28470	**NAC010**	-	y	-	-	lowly	-	-	-	-	-
AT5G67450	**ZF1**	-	y	-	-	y	-	-	-	-	-
AT3G20770	**EIN3**	-	y	-	-	y	ein3-1 more susc. (Alonso 2003)	1/3/9	2, 3 and 4	-	-
AT3G22170	**FHY3**	-	y	-	-	y	-	-	-	-	CCA1/LHY /HY5
AT3G02860	**ARS1**	y	-	y	-	y	-	-	-	-	-
AT1G68810	**ABS5**	-	-	y	-	y	-	-	-	-	-
AT3G49760	**bZIP5**	-	-	y	-	y	-	-	-	-	-
AT2G27110	**FRS3**	y	y	y	-	y	-	3	-	-	-
AT5G65640	**bHLH093**	-	y	y	-	lowly	-	-	-	-	-
AT2G33880	**HB-3**	y	y	y	-	lowly	-	-	-	-	-

Appendix 8: Reads upstream of *LHY* TSS in Arabidopsis data compared to reads upstream of the LHY homolog in rice

Reads upstream of the *LHY* TSS were viewed in IGV in all samples, ZT4.2 can be seen. These reads do not appear to correlate with regions of chromatin expected to be open and are randomly distributed along the Arabidopsis TAIR9 genome. However, reads in a sample from the rice ATAC-seq data (Wilkins *et al.,* 2016) showed a peak distribution more anticipated from ATAC-seq data. A peak can be clearly seen upstream of the rice *LHY* homolog (*OsLHY*) TSS.

To carry out this comparison a .sra file from Wilkinson *et al.,* (2016) was obtained from GEO (GEO GSE75794). This file was converted from .sra to. fastq F and R files using NCBI SRA toolkit. Fastq files were then aligned to the *O. sativa* genome (Ensembl 19 release) using Bowtie2 with the parameters previously stated for Arabidopsis data analysis. Files were converted from .sam to .bam files so alignments could be viewed in IGV.



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Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen *Botrytis cinerea*

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SUMMARY

The circadian clock, an internal time-keeping mechanism, allows plants to anticipate regular changes in the environment, such as light and dark, and biotic challenges such as pathogens and herbivores. Here, we demonstrate that the plant circadian clock influences susceptibility to the necrotrophic fungal pathogen, *Botrytis cinerea.* Arabidopsis plants show differential susceptibility to *B. cinerea* depending on the time of day of inoculation. Decreased susceptibility after inoculation at dawn compared with night persists under constant light conditions and is disrupted in dysfunctional clock mutants, demonstrating the role of the plant clock in driving time-of-day susceptibility to *B. cinerea.* The decreased susceptibility to *B. cinerea* following inoculation at subjective dawn was associated with faster transcriptional reprogramming of the defence response with gating of infection-responsive genes apparent. Direct target genes of core clock regulators were enriched among the transcription factors that responded more rapidly to infection at subjective dawn than subjective night, suggesting an influence of the clock on the defence-signalling network. In addition, jasmonate signalling plays a crucial role in the rhythmic susceptibility of Arabidopsis to *B. cinerea* with the enhanced susceptibility to this pathogen at subjective night lost in a *jaz6* mutant.

Keywords: disease resistance, plant–pathogen interaction, circadian clock, *Botrytis cinerea*, *Arabidopsis thaliana*, defence response, jasmonate, defence gene expression.

INTRODUCTION

Plants possess a robust multi-layered innate immune system to respond to attack by pathogens in their environment. The first line of defence, pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) is activated following the detection of PAMPs such as flagellin or chitin by pattern recognition receptors at the plasma membrane (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). Pathogens in turn have evolved effectors which serve both to suppress PTI and secure nutrients and water from the plant host (Macho and Zipfel, 2015). Direct or indirect detection of these effectors by plant resistance (R) proteins activates the second innate immune layer in plants, known as effector-triggered immunity (ETI) (Dangl and Jones, 2001). Finally, systemic acquired resistance, whereby infection of one part of a plant leads to increased resistance of uninfected tissues to subsequent pathogen challenge forms the final layer of innate immunity in plants (Fu and Dong, 2013). All three branches of innate immunity rely on large scale transcriptional reprogramming in the host plant, which is activated via a complex signalling network that is strongly influenced by the mutual antagonism and co-operation between jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) signalling pathways (Robert-Seilaniantz *et al.*, 2011).

Botrytis cinerea is an ascomycete necrotrophic plant pathogen with a broad host range (Williamson *et al.*, 2007). Plants are infected mainly through conidiospores which are released from infected material when disturbed, or by water splash (Choquer *et al.*, 2007; Williamson *et al.*, 2007). *B. cinerea* conidiospores adhere to and germinate on plant surfaces, forming germ tubes and appressorium-like structures within 12 h of inoculation, prior to penetration and

colonisation of the host (Gwynne-Vaughan, 1922; van Kan, 2006; Choquer *et al.*, 2007). Infectious hyphae penetrate the host tissue after enzymatic degradation of cell walls and production of H_2O_2, rather than by osmotic pressure, due to the lack of a cell wall between the appressorium and germ tube (van Kan, 2006; Choquer *et al.*, 2007). Successful infection of the plant host by *B. cinerea* is achieved through the release of non-specific phytotoxins, modification of host redox status, suppression of phytoalexin production, and subversion of programmed cell death (van Baarlen *et al.*, 2007). The chronology of the transcriptional response of Arabidopsis to *B. cinerea* has been well characterized in a fine-scale transcriptome profiling experiment, which identified groups of genes that are activated or repressed at the various stages of infection in the first 48 h after inoculation (Windram *et al.*, 2012).

Plant defences may be temporally regulated such that they are strongest at the time of maximal vulnerability to infection, e.g. against bacterial pathogens when stomata are open (Zhang *et al.*, 2013), or to coincide with the time of day when a pathogen is most abundant, or conditions are most favourable for pathogen growth. Fungal spore release is generally diurnally regulated; ascospores of *Calonectria nivalis* and *Gibberella zeae* (Sanderson, 1970; Paulitz, 1996), and *Leptosphaeria maculans* (Guo and Fernando, 2005) are released nocturnally, usually peaking around 22:00 to 24:00 h. Hartill (1980) found that ascospores of *Sclerotinia sclerotiorum* and conidiospores of *B. cinerea* were most numerous during the morning to early afternoon. The circadian clock is an endogenous time-keeping mechanism that synchronizes biological processes with the external environment, such that they occur at optimal times of the day (Dodd *et al.*, 2005). The plant circadian clock consists of a series of interlocked transcription-translation feedback loops, with negative feedback between the morning expressed transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and the evening expressed TIMING OF CAB 1 (TOC1), LUX ARHHYTHMO (LUX), EARLY FLOWERING (ELF) 3 and 4 forming the core of the clock (Hsu and Harmer, 2014). While the clock has long been known to allow plants to anticipate predictable daily changes in abiotic stimuli, such as light, only recently has it become apparent that it also allows plants to anticipate interactions with other organisms. It has been demonstrated that defence responses of Arabidopsis to both oomycete and bacterial pathogens vary with the time of day at which infection occurs (Bhardwaj *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2013). Temporal variation in susceptibility to *Pseudomonas syringae* pv *tomato* persists in Arabidopsis under constant light conditions, and is abolished in arrhythmic clock mutants (Bhardwaj *et al.*, 2011), strongly implicating the circadian clock in modulation of the host immune response.

It was recently reported that *B. cinerea* virulence is regulated by its circadian oscillator and exhibits minimal pathogenicity at dawn (Hevia *et al.*, 2015). Here, we show that plant responses to this necrotrophic fungus are also temporally regulated and play a key role in determining the outcome of infection. The plant circadian clock drives variation in susceptibility to *B. cinerea* and we hypothesised that this is achieved through the regulation of expression of key transcription factors (TFs) in the defence network, resulting in time of inoculation-dependent variation in signalling. We profiled the transcriptome of Arabidopsis leaves inoculated with *B. cinerea* at different times of day and identified a subset of TFs that are known to be direct targets of clock proteins and respond differentially to this pathogen depending on the time at which inoculation occurs. Furthermore, we show that JA/ET-responsive defence gene expression occurs more rapidly at dawn than night in response to *B. cinerea*, and identify the JA ZIM-domain protein JAZ6 as a potential link between the circadian clock and JA-mediated defence in Arabidopsis.

RESULTS

Susceptibility of Arabidopsis to *Botrytis cinerea* varies with time of inoculation under diurnal conditions

To determine whether time of inoculation affects the susceptibility of Arabidopsis to *B. cinerea* under diurnal growth conditions, we inoculated leaves from Col-0 plants with *B. cinerea* spores every 3 h over a 24 h period under 16 h light/8 h dark (LD) conditions. ANOVA revealed that susceptibility to *B. cinerea* showed significant variation with time of inoculation ($P < 0.001$), with the smallest lesion size observed after inoculation at dawn (ZT0). Susceptibility to *B. cinerea* increased following inoculations over the course of the day, and peaked after inoculation 2 h after dark (ZT18). A significant difference in lesion size on leaves inoculated at ZT0 versus ZT18 was observed at both 48 and 72 hpi (Figure 1). It should be noted that time of inoculation does not correspond to time of infection in these experiments, as spore germination and penetration of the host tissue by fungal hyphae must first occur. This process takes approximately 10–12 h from the time of inoculation, prior to which no significant differences in host gene expression are observed (Windram *et al.*, 2012).

Temporal variation in susceptibility to *B. cinerea* is driven by the circadian clock

To determine whether the temporal variation in susceptibility to *B. cinerea* is driven by the plant circadian clock we next inoculated wild-type plants from two ecotypes (Col-0 and Ws-2) with *B. cinerea* spores under free-running conditions of constant light (LL). Plants were grown for 4 weeks under standard LD conditions, and transferred to LL 24 h

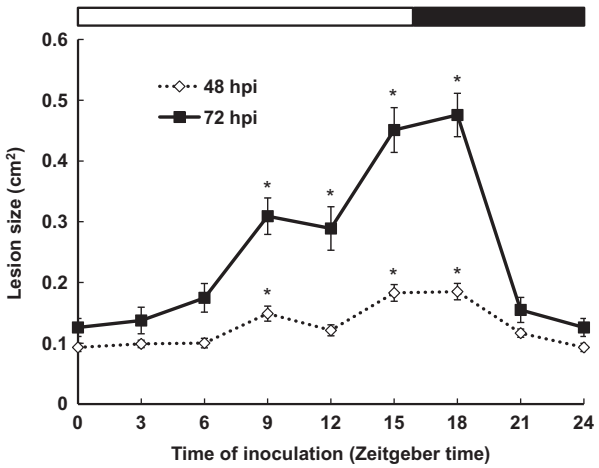

Figure 1. Arabidopsis displays temporal variation in susceptibility to *Botrytis cinerea* under diurnal conditions. Detached leaves from 4-week-old plants were inoculated with *B. cinerea* spores at dawn (ZT0) and every 3 h for 24 h under diurnal conditions, and lesion size measured at 48 and 72 h post inoculation (hpi). Data shown are mean values ± standard error of the mean (SEM) ($n = 15$) from one experiment representative of three. ANOVA revealed a significant effect of time of inoculation on lesion size ($P < 0.001$). Mean lesion sizes significantly different ($P < 0.01$) from those at ZT0 (as determined by Fisher LSD *post-hoc* analysis) are indicated by *.

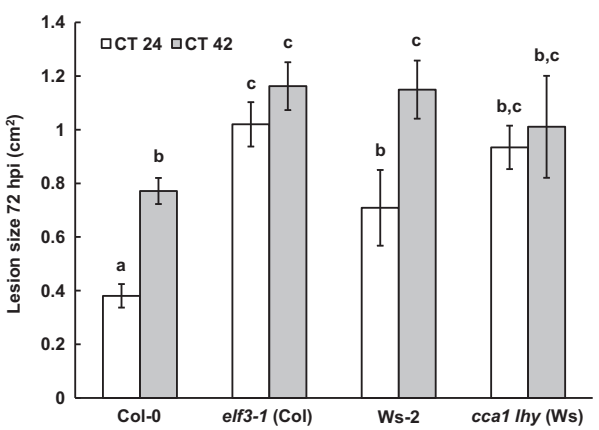

Figure 2. Arabidopsis displays clock-modulated susceptibility to *Botrytis cinerea.* Detached leaves from 4-week-old plants were inoculated with *B. cinerea* spores at CT24 (subjective dawn, white bars) or CT42 (subjective night, grey bars) under LL conditions, and lesion size measured at 72 hpi. Data shown are mean values ± standard error of the mean (SEM) from six (Col-0 and *elf3-1*) or three (Ws-2 and *cca1 lhy*) independent experiments. Twenty leaves were inoculated per genotype/time of inoculation combination in each experiment. ANOVA revealed a significant effect of both host genotype ($P < 0.001$) and time of inoculation ($P < 0.001$) on lesion size. [a–c]Mean lesion sizes with different letters are significantly different ($P < 0.01$) as determined by Fisher LSD *post-hoc* analysis.

prior to inoculation. Detached leaves were inoculated at the time points that Col-0 plants displayed the greatest resistance and susceptibility under LD conditions, i.e. subjective dawn (corresponding to circadian time 24, CT24) and subjective night (CT42). Under LL conditions, both Col-0 and Ws-2 leaves inoculated at CT24 displayed significantly smaller lesions 72 hpi than those inoculated at CT42 (Figure 2), consistent with plant clock-driven modulation of susceptibility to *B. cinerea.*

If the plant clock is responsible for the rhythmic variation in susceptibility to *B. cinerea* in Arabidopsis, then plants with dysfunctional clocks should display altered patterns of susceptibility to this pathogen, as previously reported for the bacterial pathogen *Pseudomonas syringae* (Bhardwaj *et al.*, 2011; Zhang *et al.*, 2013). We therefore examined the susceptibility to *B. cinerea* at different times of the day in two mutants with compromised clock function; *elf3-1* (in the Col-0 background) which is arrhythmic in LL, and the *cca1 lhy* double mutant (in the Ws-2 background), which has reduced amplitude, short period rhythms in LL (Mizoguchi *et al.*, 2002; Thines and Harmon, 2010). While the *elf3-1* mutant was more susceptible to *B. cinerea* than Col-0, there was no temporal variation in lesion size in this line (Figure 2). No significant difference in lesion size between leaves inoculated at CT24 versus CT42 was observed for the *cca1 lhy* double mutant either (Figure 2). This may be the result of its dramatically shortened 18 h period meaning that inoculations at CT24 and CT42 occur by chance at equivalent phases of the circadian cycle. These data provide further evidence that temporal variation in susceptibility to *B. cinerea* in Arabidopsis is driven by a functional circadian clock network.

B. cinerea growth is restricted following inoculation at subjective dawn under LL conditions

The significantly smaller lesion sizes observed after inoculation at CT24 versus CT42 in both Col-0 and Ws-2 ecotypes suggest that Arabidopsis is better able to restrict the growth of *B. cinerea* when inoculated at dawn than early night. To better quantify pathogen growth, we examined mRNA levels of the *B. cinerea* tubulin gene 72 hpi in Col-0 leaf tissue inoculated at CT24 and CT42 under LL conditions using quantitative PCR. We observed that tubulin mRNA levels, relative to those of the host gene *PUX1* (At3 g27310), whose expression is unaffected by *B. cinerea* inoculation or diurnal rhythms (Windram *et al.*, 2012), were significantly lower in plants inoculated at CT24 than at CT42 (Figure S1). Plants inoculated at subjective dawn thus appear to mount a more effective defence response to *B. cinerea* than those inoculated at subjective night.

Inoculation at dawn reduces the chance of a successful infection

Although lesion size was significantly smaller in both Col-0 and Ws-2 ecotypes when inoculated at subjective dawn versus subjective night (Figure 2), all leaves inoculated with the standard concentration of *B. cinerea* spores used in our experiments (50 000 spores mL^{-1}) developed lesions, indicating that all infections were ultimately successful. We therefore attempted to determine whether

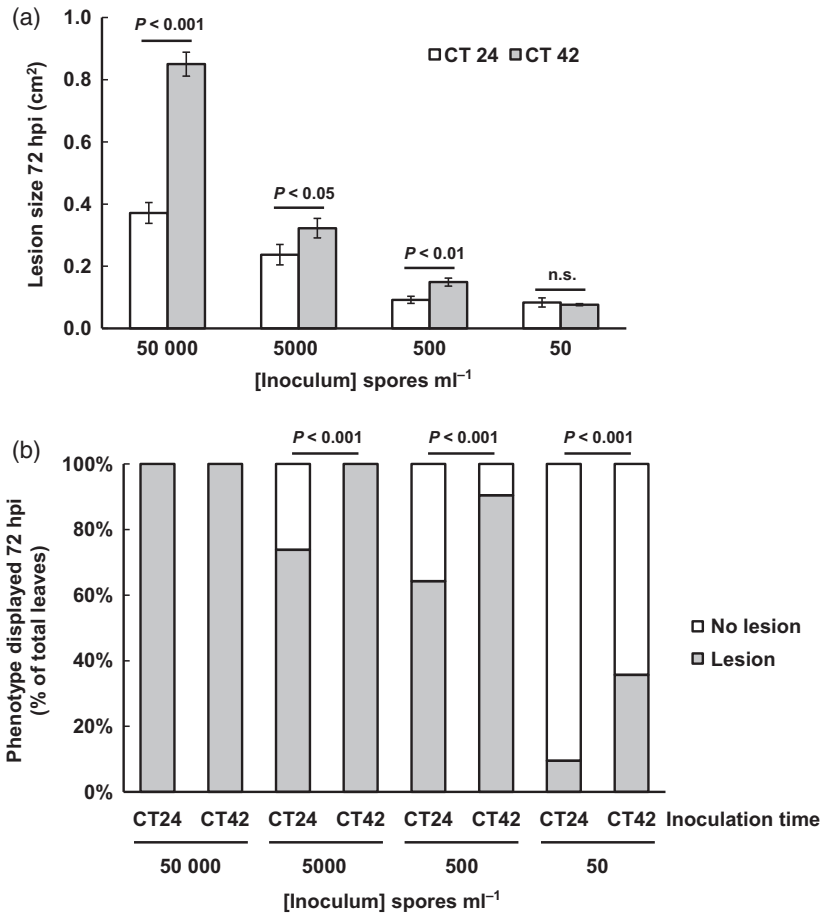

Figure 3. Inoculation at dawn reduces the chance of a successful infection. Detached leaves from 4-week-old Col-0 plants were inoculated with *B. cinerea* spores (ranging from 50 to 50 000 spores mL^{-1}) and disease symptoms assayed at 72 hpi.
(a) Mean lesion size ± standard error of the mean (SEM) on leaves that developed disease symptoms (data pooled from three independent experiments, from left to right n = 42, 42, 29, 40, 25, 26, 4 and 15 respectively), P-values from ANOVA for each comparison are indicated.
(b) Proportion of leaves displaying disease symptoms (determined as formation of primary lesion) as percentage of total number of leaves inoculated across the three experiments (n = 42). The P-values shown are from chi-squared analysis testing for a significant relationship between time of inoculation and success of infection at each spore concentration.

clock-modulation of the defence response might confer a fitness advantage at lower spore inocula concentrations by preventing the development of disease symptoms. As expected, a reduction in lesion area 72 hpi was observed as spore concentration of the inoculum was reduced under LL conditions, though temporal variation in susceptibility was still observed at all spore concentrations tested except 50 spores mL^{-1} (Figure 3a). However, as we observed that not all leaves developed lesions at spore concentrations below 50 000 mL^{-1}, we also scored for the presence or absence of lesion formation at 72 hpi. The frequency of successful infections decreased with reducing spore concentration, but was consistently higher on leaves inoculated at CT42 (Figure 3b). Chi-squared analysis revealed a highly significant relationship between time of inoculation and the frequency of disease symptoms at all three lower spore concentrations (Figure 3b) again indicating that a more effective defence response is launched after inoculation at dawn.

The host transcriptional response to *B. cinerea* varies with time of inoculation

Large-scale transcriptional reprogramming following pathogen detection is a major component of the host immune response, with differential expression of approximately one-third of the Arabidopsis genome occurring within 48 h of inoculation with *B. cinerea* 6 h after dawn (Windram *et al.*, 2012). One possible explanation for the temporal variation in susceptibility to *B. cinerea* in Arabidopsis is that the magnitude and/or speed of the transcriptional defence response to this pathogen varies over a 24 h period. To test this hypothesis, we analysed global gene expression using NimbleGen arrays following inoculation of Arabidopsis leaves under LL conditions at either subjective dawn (CT24) or night (CT42). Based on recent high-resolution expression profiling of the Arabidopsis transcriptome response to *B. cinerea*, we selected 18 and 22 hpi for gene expression analysis as these were time points at which a large proportion of genes first showed differential expression (Windram *et al.*, 2012).

As an initial validation of the microarray data, we analysed the expression of 31 marker genes consistently upregulated in response to *B. cinerea* infection in previous studies (Dataset S1). Of these 31 genes, 27 showed a ≥1.5-fold ($log_2$0.6) up-regulation in response to *B. cinerea* in at least one of the two time points after inoculation at subjective dawn or subjective night, indicating that the infections had triggered the expected transcriptional response in the

host. The fact that these studies were carried out on different platforms, and the current study was limited to two time points after inoculation may explain why the remaining four genes were not upregulated as expected.

From the expression data, we identified 901 genes that are responding differentially to inoculation with *B. cinerea* at dawn compared with night (Dataset S2). All 901 differentially expressed genes (DEGs) are significantly up- or downregulated in response to *B. cinerea*, but in addition show a significant difference in expression in leaves inoculated at subjective dawn compared with leaves inoculated at subjective night, and/or a significant difference in their response to infection (i.e. fold change in infected versus mock inoculated leaves) at subjective dawn compared to night (see Figure S2).

Transcription factor genes show both rhythmic basal expression and differential sensitivity to *B. cinerea* infection

Members of a number of TF families, including WRKY, AP2/ERF, NAC and MYB, have been shown to play a role in defence against *B. cinerea* (Birkenbihl and Somssich, 2011). Furthermore, TFs form the backbone of the gene regulatory network mediating transcriptional reprogramming in response to *B. cinerea* infection (Windram *et al.*, 2012). As such, temporal variation in TF expression could have a significant impact on the host defence response. Of the 901 genes that show differential expression after inoculation at different times of the day, 99 encode TFs (Dataset S3). These differentially expressed TFs include the known positive regulators of *B. cinerea* defence responses, MYB108 (Mengiste *et al.*, 2003) and ERF6 (Moffat *et al.*, 2012). Up-regulation of both TFs was greater at 18 hpi in leaves inoculated at subjective dawn compared with night, indicative of a faster activation of the defence regulatory network following inoculation at dawn.

We investigated whether the more rapid activation of the defence network was a result of differential basal expression of key TFs such that the same fold change in expression following infection results in significantly different absolute expression levels (expression level-only genes in Dataset S3 and Figure S2b) or whether there was differential induction/repression of TF genes after inoculation at subjective dawn versus subjective night leading to different expression levels (both a fold change and expression level difference in Dataset S3 and Figure S2c). This latter situation can be interpreted as gating of the defence response by the clock so that the magnitude of the host response to pathogen detection varies with time of day. We found evidence for both types of regulation in up- and downregulated TF genes (Figure S3). We also found TFs that had a different fold change in response to infection at subjective dawn or night but the resulting expression levels were not significantly different (fold change only in Dataset S3). This suggests that the level of expression of these genes is tightly controlled and/or there is a maximal level of expression for these genes. We selected four of the differentially expressed TFs for validation of their expression profiles using qPCR. The qPCR analysis, also carried out on samples from plants inoculated under LL conditions, confirmed the differential expression of these TFs after inoculation at subjective dawn or night (Figure 4). Enhanced expression of TF genes after inoculation at dawn appears to be driving a more rapid host defence response, potentially leading to the observed decrease in susceptibility to *B. cinerea* infection.

Approximately half of the TFs differentially expressed after inoculation at subjective dawn or night show circadian expression patterns in the data sets analysed by Hazen *et al.* (2009) or Covington *et al.* (2008), which may drive differences in basal expression in these genes. Strikingly, the differentially expressed TFs are highly enriched for genes that are direct targets of the core clock components (Dataset S3). Direct targets of TOC1 (Huang *et al.*, 2012), PRR5 (Nakamichi *et al.*, 2012), and PRR7 (Liu *et al.*, 2013) were significantly enriched ($P < 0.0001$) compared with the genome as a whole indicating a high level of input from the clock on these defence-related regulators.

Hormone responsive genes are differentially expressed after inoculation at dawn or night

To gain insight on the biological processes represented by the genes differentially responding to inoculation at dawn or night, we grouped the 901 DEGs into eight groups on the basis of their expression patterns (Dataset S2). The first four groups are: (i) UPUP18 – genes upregulated in response to infection and with a higher expression level and/or induction after inoculation at dawn compared to night by 18 hpi; (ii) UPUP22 – genes upregulated in response to infection and with a higher expression level and/or induction after inoculation at dawn compared to night only at 22 hpi; (iii) and (iv) the corresponding downregulated groups, DOWNDOWN18 (genes downregulated in response to infection and with a lower expression level and/or greater repression after inoculation at dawn compared to night by 18 hpi) and DOWNDOWN22 (lower expression and/or greater repression after inoculation at dawn compared with night only observed at 22 hpi). These four groups contained the majority (71%) of the DEGs: UPUP18 – 219 DEGs, UPUP22 – 44 DEGs, DOWNDOWN18 – 257 DEGs, DOWNDOWN22 – 123 DEGs. These appear to be genes whose response to infection is amplified or occurs more rapidly after inoculation at dawn compared to night and hence may underlie the enhanced defence response after inoculation at dawn.

The remaining 246 DEGs showed a less intuitive expression pattern. Forty-seven and 98 DEGs were up-regulated in response to infection, but showed lower expression or

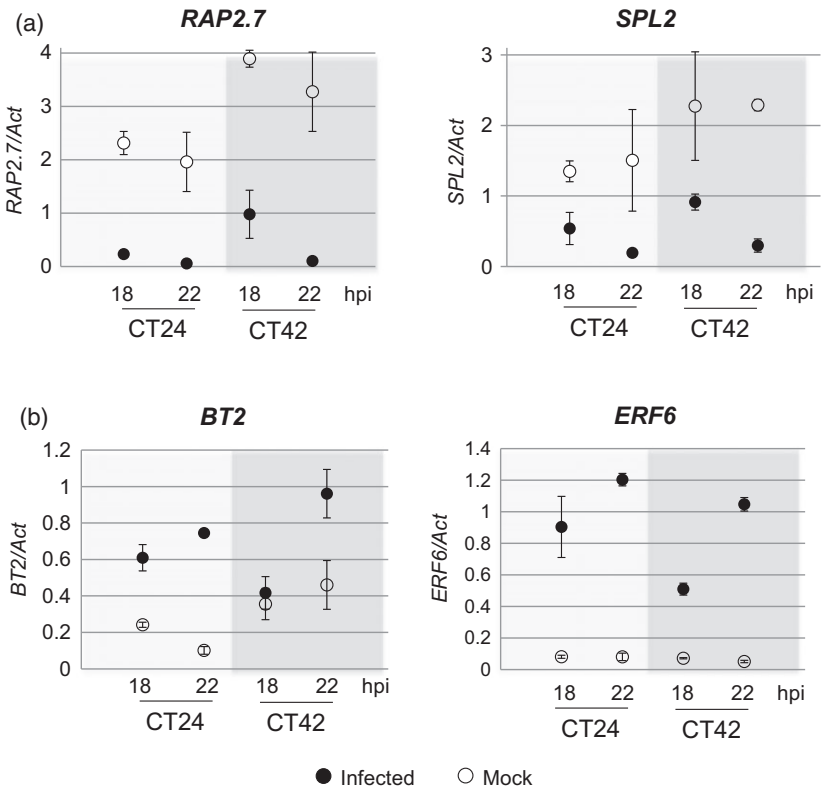

Figure 4. Differential expression (as determined by qPCR) of transcription factor (TF) encoding genes in response to infection at subjective dawn and night under LL conditions.
(a) TF genes showing different basal levels of expression at CT24 (subjective dawn) or CT42 (subjective night), but a similar fold change in response to infection and (b) TF genes with a different fold change in response to infection at CT24 or CT42. Filled circles are expression values after *B. cinerea* infection and open circles from mock inoculated leaves. Values shown are mean expression values (normalized to *Actin2* expression) from three biological replicates ± standard error of the mean (SEM).

reduced response to infection after inoculation at subjective dawn compared with night at 18 or 22 hpi respectively (UPDOWN18, UPDOWN22). Similarly 53 and 48 DEGs were downregulated in response to infection but showed higher expression or response to infection after inoculation at subjective dawn compared to night at 18 and 22 hpi respectively (DOWNUP18, DOWNUP22).

We focused on the four main groups of genes with enhanced response to inoculation at subjective dawn and investigated the biological processes these genes are involved in using enrichment of Gene Ontology (GO) terms (Ashburner *et al.*, 2000) (Figure 5 and Dataset S4). This analysis suggested that the majority of functionally coordinated changes in gene expression occur by 18 hpi; very few GO terms were enriched in genes only showing a difference in expression between subjective dawn and night inoculation at 22 hpi. The presence of the GO term 'circadian rhythm' in the genes repressed in response to infection is expected and an initial validation of our expression profiling approach. We know that the amplitude of core clock gene expression is reduced in response to *B. cinerea* infection (Windram *et al.*, 2012) and that these genes are obviously expressed at different basal levels at dawn and night.

Several of the terms in Figure 5 (including toxin catabolism and glucosinolate biosynthesis) match those identified in our previous expression time series following inoculation of Arabidopsis leaves with *B. cinerea* 6 h after dawn (Windram *et al.*, 2012) suggesting that a specific sector of the defence response is temporally regulated.

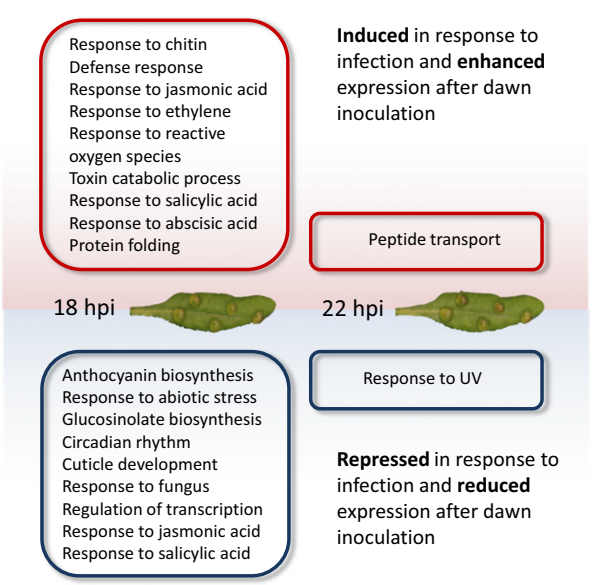

Figure 5. Biological processes associated with the enhanced defence response at subjective dawn. The figure indicates selected Gene Ontology (GO) biological process terms enriched in genes whose response to infection is enhanced at subjective dawn compared with subjective night.

A striking observation is the abundance of terms associated with hormone signalling and response pathways both in the groups induced and repressed in response to infection, as well as terms directly related to the perception and response to fungal infection. Notably, genes responding to four phytohormones (ET, JA, SA and ABA) are enriched in upregulated genes showing an enhanced response to infection by 18 hpi at dawn compared to night (UPUP18).

We used the MEME-LAB software (Brown *et al.*, 2013) to determine whether the promoters of genes responding differentially to infection at different times of the day were enriched for particular DNA sequences. The MEME-LAB results are given in Dataset S5 but of particular interest was a motif enriched in the promoters of genes in the UPUP18 group. This motif, a GCC-box motif, was found in the promoters of five genes, three of which were plant defensins (*PDF1.2*, *1.2b* and *1.2c*). The GCC-box has been identified as a binding site for a number of AP2/Ethylene response factor (ERF) TFs and synergistic JA/ET signalling is known to converge on the GCC-box (Zarei *et al.*, 2011). Two other plant defensins (*PDF1.1* and *PDF1.3*) were also in the UPUP18 group and using the position weight matrix for the GCC-box found by MEME-LAB, a motif similar to the GCC-box was identified in the upstream promoter sequence of *PDF1.3* (ATCATCAGCCCA). We analysed the expression of *PDF1.1* and *PDF1.3* following inoculation with *B. cinerea* at subjective dawn and night using qPCR (Figure 6). As expected, both genes were induced in response to *B. cinerea* infection and, as seen in our array data, their expression was significantly higher at 18 hpi (and for *PDF1.3* also at 22 hpi) in plants inoculated at subjective dawn compared with subjective night (Figure 6). These results suggest that activation of JA/ET-mediated defence gene expression is indeed more rapid in plants inoculated at dawn.

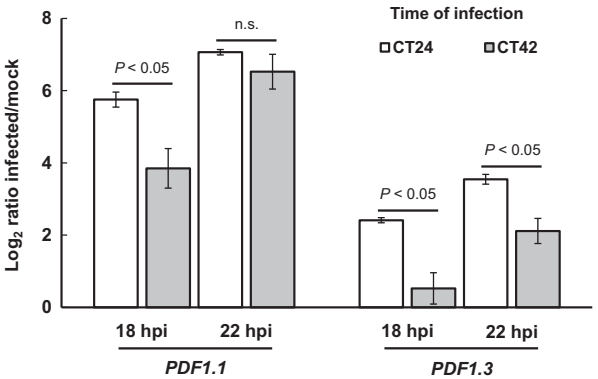

Figure 6. Differential induction of *PDF* gene expression in response to inoculation at subjective dawn versus night. Detached leaves were inoculated with *B. cinerea* spores or mock inoculated at CT24 or CT42 under LL conditions, and harvested at 18 and 22 hpi for RNA extraction. Relative expression of *PDF1.1* and *PDF1.3* was determined by qPCR (with normalization to *Actin2* levels) and $\log_2$ expression ratios calculated by normalizing *PDF*/*Act2* values from infected leaves to those in mock inoculated controls for each time point. Values are mean $\log_2$ expression ratios (± SEM) from three biological repeats, and *P*-values shown are from one-way ANOVA testing for a significant difference in induction between leaves inoculated at CT24 versus CT42. n.s., not significant.

Jasmonate signalling is required for circadian-driven variation in *B. cinerea* susceptibility

Jasmonate ZIM-domain (JAZ) proteins are transcriptional repressors that act as negative regulators of JA and ET-mediated defence responses, through their interaction with COI1, and the MYC2/3/4 and EIN3/EIL1 TFs (Chini *et al.*, 2007; Fernández-Calvo *et al.*, 2011; Zhu *et al.*, 2011). Upon perception of the JA-Ile conjugate, COI1 interacts with JAZs targeting them for degradation, thereby relieving their repressive effect on MYC and EIN3/EIL1-mediated gene expression (Thines *et al.*, 2007). To determine whether JA signalling might play a role in clock-modulated susceptibility to *B. cinerea*, we analysed lesion formation in *jaz* mutants, reasoning that JAZ-mediated repression of JA signalling would be compromised in these plants. Because of potential redundancy in JAZ function, we began by analysing lesion formation in *jaz* triple mutants in which redundancy may be overcome. In contrast to Col-0, temporal variation in susceptibility to *B. cinerea* was not observed in the *jaz5,6,10* triple mutant under LD conditions (Figure 7a). However, *jaz5,7,10* displayed the normal enhanced susceptibility after inoculation at night. The difference between these two triple mutants suggested that *JAZ6* could be a key player in circadian regulation of defence against *B. cinerea.* Furthermore, the expression of the *JAZ6* gene is markedly induced in response to *B. cinerea* infection (Figure S4). Consistent with this hypothesis, the single *jaz6* mutant did not show a significant time-of-day difference in lesion size after inoculation at dawn or night (Figure 7a). This lack of temporal variation in susceptibility in the *jaz6* and triple *jaz5,6,10* mutants was also evident under LL conditions (Figure 7b). These results indicate that JAZ6 is required for circadian-driven variation in susceptibility to *B. cinerea* and may be responsible for repression of JA/ET-responsive genes after inoculation at subjective night.

DISCUSSION

The plant circadian clock influences the outcome of *Botrytis cinerea* infections

We have demonstrated that Arabidopsis infection by the necrotrophic pathogen *B. cinerea* leads to different outcomes, depending on the time of the day at which plants are inoculated with fungal spores. Under diurnal (LD) conditions lowest susceptibility to *B. cinerea* was observed in leaves inoculated at dawn (ZT0) (Figure 1). Thereafter susceptibility to this pathogen increased during the light period, and peaked when leaves were inoculated

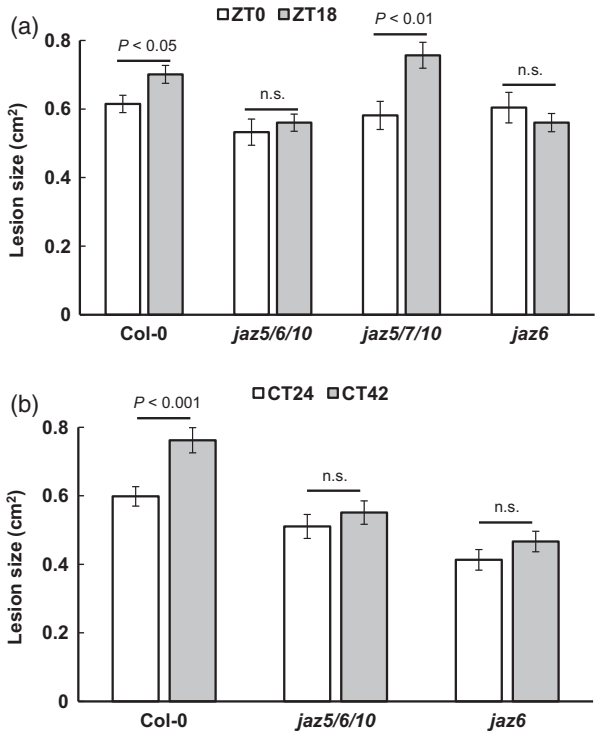

Figure 7. Temporal variation in susceptibility to *B. cinerea* requires JAZ6. Detached leaves from 4-week-old plants were inoculated with *B. cinerea* spores under: (a) LD at dawn or night (ZT0 and ZT18) or (b) LL at subjective dawn or night (CT24 and CT42) conditions and lesion area measured at 72 hpi. Data shown are mean lesion sizes ± standard error of the mean (SEM) on wild-type (Col-0) leaves versus *jaz* mutants ($n \geq 30$ for each time of inoculation/genotype combination). ANOVA was used to test whether lesion size was significantly different between leaves inoculated at dawn versus night; *P*-values for each comparison are shown. n.s., not significant.

18 h after dawn, 2 h into the dark period (ZT18). This variation in host susceptibility could result from differences in pathogen virulence and/or the host defence response over the course of the day.

Light has previously been reported to negatively affect the virulence of *B. cinerea*, with reduced lesion sizes reported on Arabidopsis leaves infected under LL versus LD conditions (Canessa *et al.*, 2013). However, we found differences in lesion sizes between inoculations carried out at different times during the light period, for example ZT0 versus ZT9, 12 or 15, as well as the dark period, ZT18 versus ZT21; whereas lesion sizes following inoculations at ZT0 or ZT3 in the light were not significantly different from those at ZT21 in the dark (Figure 1). These results indicate that the time-of-day variation in the success of *B. cinerea* infection is not due simply to the presence or absence of light. A recent study demonstrated that the *B. cinerea* circadian clock contributes to the outcome of infection, and the pathogen was shown to have maximal virulence to Arabidopsis plants when inoculated at dusk (Hevia *et al.*, 2015). Although conidiation is promoted by light in a number of *B. cinerea* strains (Canessa *et al.*, 2013), the *B. cinerea* pepper isolate used in our experiments produces conidia irrespective of the presence of light. Crucially, in our experiments the pathogen was cultured in constant darkness, which provided no entrainment for the clock, and was exposed to light only when the conidiospores were removed immediately prior to use in inoculations, after which infections developed in constant light. Thus, the pathogen was in an equivalent state at each inoculation time and unlikely to be the cause of the time-of-day variation observed in Figure 1. Instead our observations that the time of inoculation variation in susceptibility to *B. cinerea* persists under constant light (LL) conditions in both Col-0 and Ws ecotypes, but not in dysfunctional clock mutants in these genetic backgrounds, strongly suggest that temporal variation in resistance to *B. cinerea* infection is mediated by the plant circadian clock. The demonstration that mutation of a single gene, *JAZ6*, causes loss of time-of-day variation in susceptibility under both LD and LL conditions again indicates that the outcome of infection is driven by plant mechanisms.

Comparison of rhythmic defence against biotrophic and necrotrophic pathogens

We have previously shown that Arabidopsis plants pressure-inoculated with *P. syringae* display maximal resistance to this pathogen in the subjective morning and are most susceptible during the subjective night (Bhardwaj *et al.*, 2011). Given the different lifestyles of *B. cinerea* and *P. syringae* (necrotroph and hemi-biotroph), and the documented antagonism between hormonal defence pathways against these pathogens (Koornneef and Pieterse, 2008), it may seem surprising that a similar temporal pattern of host resistance was observed. However, while infiltration of *P. syringae* directly into the apoplast results in a host transcriptional response within 2 h (Truman *et al.*, 2006), no significant differences in host gene expression were observed in Arabidopsis leaves inoculated with *B. cinerea* spores until 10 hpi (Windram *et al.*, 2012). This delay is likely because *B. cinerea* conidiospores must germinate in the inoculation droplet and subsequently form the infectious hyphae required for enzymatic degradation of the host cell wall and penetration of the host. Thus plants inoculated with *B. cinerea* spores at CT24 may only respond to the pathogen at around CT34 (late subjective day), and those inoculated at CT42 at around CT52 (subjective morning). If so, then the true times of maximal resistance to *P. syringae* and *B. cinerea* would be 12 h out of phase, mirroring clock-modulated SA and JA levels which peak during the middle of the subjective night and day respectively in uninfected plants under constant dark conditions (Goodspeed *et al.*, 2012). Similarly under LD conditions, genes associated with JA signalling peak 10–12 h after

dawn, again coinciding with the first plant responses to *B. cinerea* inoculation at dawn (Windram *et al.*, 2012).

Mechanism of clock-driven defence

How does the circadian clock influence host susceptibility to *B. cinerea?* Without knowing the biology of *B. cinerea*, an obvious suggestion would be that clock regulation of stomatal aperture (Dodd *et al.*, 2005) could influence entry of the pathogen to the host. The opportunistic use of open stomata and wounds has been reported during infection by *B. cinerea* (Fourie and Holz, 1995), although the primary route of entry is via penetration of the host cuticle (Williamson *et al.*, 2007). We examined hyphal behaviour following spore germination on Arabidopsis leaves and found no evidence of directional growth of *B. cinerea* hyphae towards the stomata (Figure S5a). Indeed, on several occasions we observed hyphae growing over an open stomate (Figure S5b). Hence, it does not appear that stomata are important sites of entry into Arabidopsis for *B. cinerea*, and the temporal variation in immunity to this pathogen is unlikely to be explained by clock-driven changes in stomatal aperture.

Our results suggest that the difference in susceptibility of Arabidopsis to *B. cinerea* at different times of the day is mediated by differential activation of the plant defence response with preferential induction of the defence response following inoculation at dawn. We identified a set of genes that exhibited an enhanced response to inoculation at subjective dawn compared with night and are involved in processes previously linked to the plant defence response against *B. cinerea* (Figure 5; Windram *et al.*, 2012). Our data suggest that activation of, and/or flow of information through, the defence regulatory network is enhanced after inoculation at subjective dawn compared with at night. Several known key regulators of susceptibility to *B. cinerea* were more rapidly induced or repressed in response to inoculation at subjective dawn than night, including *ERF1* (Berrocal-Lobo *et al.*, 2002); *ERF6* (Moffat *et al.*, 2012); *MYB108* (Mengiste *et al.*, 2003) and *WRKY60* (Xu *et al.*, 2006). TFs responding differentially to inoculation at dawn compared to night were highly enriched for known direct targets of core clock TFs, suggesting direct links between the circadian clock and the defence regulatory network.

Role of the jasmonate pathway

It is well known that the plant hormone JA influences the outcome of infection by *B. cinerea* (Thomma *et al.*, 1998) and it was clear from our transcriptome profiling that it plays a key role in the time-of-day variation of susceptibility to *B. cinerea.* JA biosynthesis and signalling are known to be under the control of the circadian clock and clock-driven accumulation of JA during the day was shown to contribute to protection against grazing by the herbivore *Trichoplusia ni* (Goodspeed *et al.*, 2012). Furthermore, the clock component TIME FOR COFFEE (TIC) mediates rhythmic expression of the JA receptor *COI1* under diurnal conditions and temporal gating of at least some JA responses (Shin *et al.*, 2012). TIC interacts directly with MYC2 preventing accumulation of this TF (Shin *et al.*, 2012) which is also regulated by binding of JAZ repressors (Chini *et al.*, 2007).

We have identified JAZ6 as a crucial component in of time-of-day defence against *B. cinerea* and suggest that JAZ6 is another important link between the clock and JA signalling pathways. JAZ proteins regulate JA responses by forming a repressive complex with various TFs and the co-repressor Topless, often via the adapter protein NINJA (Pauwels *et al.*, 2010) although JAZ6 contains the repressive EAR domain suggesting it may independently repress transcription. After binding of activated JA (JA-Ile) to the JA receptor COI1, JAZ proteins are targeted for degradation by the 26S proteasome (Thines *et al.*, 2007) relieving repression on the bound TFs thereby resulting in activation of downstream JA processes. We would expect the absence of *JAZ6* to relieve repression of a positive regulator of defence leading to decreased susceptibility at night. Why does JAZ6 only exert its effect at night? *JAZ6* expression is under circadian control (Covington *et al.*, 2008; Hazen *et al.*, 2009) but its induction in response to *B. cinerea* infection is significantly higher (Figure S4). Future measurement of JAZ6 protein levels, and its presence in repressive defence-related TF complexes, will indicate whether temporal variation in JAZ6 accumulation or activity is responsible for restricting its effects on the defence response to night.

Adaptive significance of time-of-day variation in defence

We propose two hypotheses to explain the time-of-day variation in susceptibility to pathogens in plants. Plants may anticipate an increased likelihood of infection at dawn or circadian regulation of plant processes (such as metabolic pathways) for non-defence-related reasons may result in the defence response being sub-optimal at night. *B. cinerea* may have exploited such a temporal gap in plant defences and used its own circadian clock to align attack strategy with times of plant least resistance (Hevia *et al.*, 2015). There is evidence to support the first anticipatory hypothesis. Circadian and diurnal rhythms of spore development or release have been noted in many fungal genera (Ingold, 1971) with these spore release rhythms persisting in some species even in constant laboratory conditions (Pittendrigh *et al.*, 1959; Ingold, 1971; Canessa *et al.*, 2013) indicating endogenous control. The morning reduction in humidity and increase in temperature causes release of *B. cinerea* conidia into the air (Williamson *et al.*, 2007) resulting in greatest spore abundance during the morning to early afternoon (Hartill, 1980). The likelihood of

successful infection of Arabidopsis leaves by *B. cinerea* was lower following inoculation at subjective dawn than subjective night (Figure 3b), consistent with the idea that plant defences are enhanced in anticipation of the abundance of spores in the morning. Further understanding of how the plant clock is driving a more effective defence response after inoculation at dawn will help in pinpointing crucial regulatory components that could be used to enhance resistance against this economically important fungal pathogen.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis seeds were sown on a 1:1 mix of peat (Jiffy Products, Norway, http://www.jiffygroup.com/) and vermiculite and stratified for 48 h at 4°C in the dark. Plants were grown under a long-day photoperiod (16 h light, 8 h dark) at 22°C and 55% relative humidity, and cool white fluorescent light of 80–100 μmol m^{-2} sec^{-1}. Where plants were to be infected with *B. cinerea* under constant light (LL), they were transferred to LL conditions during the 16 h light period 24 h prior to inoculation. The *cca1-11 lhy-21* double mutant (N9809) was obtained from the Nottingham Arabidopsis Stock Centre. The *elf3-1* mutant (Zagotta *et al.*, 1992) was a kind gift from Frank Harmon. Single and triple *jaz* mutants are described in de Torres Zabala *et al.* (2015). *JAZ6* mRNA levels 18 hpi with *B. cinerea* were greatly reduced in the *jaz6* mutant relative to those observed in wild-type plants (Figure S6).

Botrytis cinerea inoculations

Botrytis cinerea pepper isolate (described in Denby *et al.*, 2004) was sub-cultured on apricot halves in the dark at 25°C 2 weeks prior to use of the spores. To determine the susceptibility of Arabidopsis to *B. cinerea* at each time of inoculation detached leaves were inoculated with 10 μL of half-strength grape juice containing 5×10^4 spores mL^{-1}, as previously described (Ingle and Roden, 2014). Lesions were photographed 48–72 h post inoculation (hpi), and lesion area determined using IMAGEJ (http://rsbweb.nih.gov/ij/). For microarray experiments, leaf five was detached from 4-week-old wild-type (Col-0) plants, inoculated with six 10 μL droplets of the spore solution (spaced evenly over the leaf surface) at CT24 (subjective dawn) or CT42 (subjective night), and harvested 18 or 22 hpi. For all experiments half-strength grape juice served as the mock infection control. Lactophenol–trypan blue staining (Koch and Slusarenko, 1990) was used for microscopic visualisation of hyphal structures following conidiospore germination.

NimbleGen microarray analysis

Total RNA was extracted from four single Arabidopsis leaves using Trizol (Ambion, ThermoFisher Scientific, https://www.thermofisher.com/za/en/home.html), and cleaned up using the RNeasy kit (Qiagen, https://www.qiagen.com/za/) with on-column DNase digestion. Equal amounts of total RNA were pooled from the four biological replicates for each of the eight experimental treatments (18 hpi at CT24, 22 hpi at CT24, 18 hpi at CT42 or 22 hpi at CT42 with *B. cinerea* spores or half-strength grape juice) and 100 ng of each pool amplified using the MessageAmp II kit (Life Technologies). Double-stranded cDNA was synthesised from 2.5 μg of the resulting aRNA using the Superscript Double-stranded cDNA synthesis kit (Life Technologies). Following RNase A digestion, ds cDNA was purified using Qiaquick columns (Qiagen). Five hundred ng of ds cDNA was labelled using the NimbleGen One-Color DNA labelling kit (Roche, https://lifescience.roche.com/shop/home), as per the manufacturer's instructions except that half volumes of all reagents were used for all steps. Four μg of the resulting Cy3-labelled cDNA was hybridised against NimbleGen *A. thaliana* gene expression 12 × 135K arrays custom designed for the TAIR10 *Arabidopsis thaliana* genome annotation (Design ID OID37507), two duplicate arrays per sample, which were washed and scanned using the NimbleGen MS 200 scanner as per the manufacturer's instructions. The RMA algorithm (Irizarry *et al.*, 2003) was used to normalize the expression data and generate $\log_2$ expression values for each gene. All raw and normalized microarray data has been deposited in GEO (GSE70137). A three-factor ANOVA was performed to identify genes showing significant expression differences due to infection with *B. cinerea* (I), time of day of inoculation (ToD), time after inoculation (T) and/or interaction between these factors, and a Bonferroni multiple testing correction applied. Genes whose expression showed a significant effect of I × ToD, I × ToD × T, or a significant effect of I and ToD, or I and ToD × T were selected and filtered to only keep genes showing a $\geq \log_2 0.6$ change in expression due to *B. cinerea* infection at any time point. Further filtering was done to select genes showing a $\geq \log_2 0.6$ difference in expression between infected samples inoculated at dawn compared with night, and/or a $\geq \log_2 0.6$ difference in the ratio of infected:mock at dawn compared with night.

Gene Ontology and promoter sequence motif analysis

The DEG were grouped depending on their expression profiles (see Methods S1) and analysed for overrepresented Gene Ontology (Ashburner *et al.*, 2000) terms. The same groups were tested for overrepresentation of known TF binding motifs in their upstream promoter sequences as outlined in Breeze *et al.* (2011).

Quantitative PCR analysis

Quantitative PCR was performed using a RotorGene RG3000A instrument (Corbett Research, Australia, https://www.qiagen.com/za/corbett/welcome.aspx). The cDNA for these experiments was synthesised from 1 μg of total RNA for each independent biological replicate (leaf 5 from a single Col-0 plant) using Superscript III reverse transcriptase (Life Technologies). The qPCR reactions consisted of 1 μL template cDNA, 5 μL Kapa SYBR FAST Universal 2× qPCR Master Mix (Kapa Biosystems, South Africa, https://www.kapabiosystems.com/), and 200–900 nM of each primer in a final volume of 10 μL. Amplification conditions were as follows; an initial step at 95°C for 3 min, followed by 40 cycles of 95°C for 3 sec, primer annealing at 58 or 60°C for 20 sec and elongation at 72°C for 1 sec. The relative expression level of each gene of interest was calculated with the ROTORGENE 6000 (Corbett Life Science, https://www.qiagen.com/za/corbett/welcome.aspx) series software v1.7 using the two standard curve method. Details of the primers used and specific qPCR reaction conditions can be found in Table S1. *Actin2* (At3 g18780) and *PUX1* (At3 g27310) were used to normalize expression data in these experiments as mRNA levels of these genes are unaffected by *B. cinerea* infection (Windram *et al.*, 2012), and constant across our mock inoculation microarray samples.

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AUTHOR CONTRIBUTIONS

RAI, LCR, IAC and KJD had the original ideas for the research. RAI, CS, WS, NA, RS and MG carried out experiments. All authors had input into the design of experiments and data analysis. RAI, LCR and KJD wrote the manuscript with contributions from all authors.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Growth of *Botrytis cinerea* is restricted in plants inoculated at subjective dawn versus subjective night.

Figure S2. Diagram illustrating selection of differentially expressed genes.

Figure S3. Differential expression of transcription factor (TF) encoding genes in response to infection at subjective dawn or night under LL conditions.

Figure S4. *JAZ6* expression is transiently induced during *B. cinerea* infection.

Figure S5. Stomata are not a primary point of entry for *B. cinerea* hyphae during infection of Arabidopsis.

Figure S6. Expression of *JAZ6* in the *jaz6* mutant line and Col-0 18 hpi with *B. cinerea* or mock control.

Table S1. Primers used in quantitative PCR experiments.

Methods S1. Grouping of the differentially expressed genes for Gene Ontology and motif analysis.

Dataset S1. Expression data 18 and 22 hpi after subjective dawn and night inoculations with *B. cinerea* for genes with consistent expression patterns in previous studies.

Dataset S2. Arabidopsis genes whose expression changes significantly in response to *B. cinerea* and to the time of day at which inoculation occurred.

Dataset S3. Transcription factor genes that are differentially expressed in response to inoculation with *B. cinerea* at different times of the day.

Dataset S4. Biological Process Gene Ontology terms significantly overrepresented in groups of genes differentially expressed in response to inoculation at different times of the day.

Dataset S5. Motifs significantly overrepresented in upstream regions of groups of genes differentially expressed in response to inoculation at different times of the day.

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